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(54) Title: SEMI-SYNTHETIC SAPONIN ANALOGS WITH CARRIER AND IMMUNE STIMULATORY ACTIVITIES FOR DNA AND RNA VACCINES

(57) Abstract: The present invention discloses novel saponin derivatives for use with nucleic acids that induce an immune response when administered to animals and humans. The novel saponin derivatives disclosed comprise (a) a saponin aglycone core, wherein the aglycone core is covalently linked to one or more oligosaccharide chains; (b) a positively charged cationic chain, and optionally (c) a naturally occurring or synthetic lipophilic chain. Pharmaceutical and veterinary compositions comprising one or more of the novel saponin derivatives and saponin derivative/polynucleotide complexes are also disclosed. Disclosed as well are methods of using the novel saponin derivatives to deliver a polynucleotide molecule to cells of an animal, to stimulate or generate an immune response in an animal, and to generate a detectable immune response in an animal.

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SEMI-SYNTHETIC SAPONIN ANALOGS WITH CARRIER AND IMMUNE STIMULATORY ACTIVITIES FOR DNA AND RNA VACCINES

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention is in the field of nucleic acid and antisense nucleic acid delivery into cells. More particularly, the invention pertains to novel saponin derivatives for use with nucleic acids that induce an immune response when administered to animals and humans.

Background Art

[0002] DNA and RNA vaccines are the terms broadly used to describe methods of transiently transfecting cells with DNA plasmids or mRNA encoding for protein antigens whose expression stimulates an immune response. Because of the intracellular production of these antigens and their processing by the endogenous pathway, nucleic acid vaccines elicit humoral as well as T-cell immunity with cytotoxic T lymphocytes (CTL) production.

[0003] The immune system may exhibit both specific and nonspecific immunity (Klein, J., *et al., Immunology (2nd)*, Blackwell Science Inc., Boston (1997)). Generally, specific immunity is produced by B and T lymphocytes, which display specific receptors on their cell surface for a given antigen. The immune system may respond to different antigens in two ways: 1) humoral-mediated immunity, which includes B cell stimulation and production of antibodies or immunoglobulins (other cells, however, are also involved in the generation of an antibody response, e.g. antigen-presenting cells (APCs, including macrophages) and helper T cells (Thl and Th2)), and 2) cell-mediated immunity (CMI), which generally involves T cells, including cytotoxic T lymphocytes (CTLs), although other cells are also involved in the generation of a CTL response (e.g., Thl and/or Th2 cells and APCs).

[0004] Nonspecific immunity encompasses various cells and mechanisms such as phagocytosis (the engulfing of foreign particles or antigens) by macrophages or granulocytes, and natural killer (NK) cell activity, among others. Nonspecific immunity relies on mechanisms less evolutionarily advanced (e.g., phagocytosis, which is an important host defense mechanism) and does not display the acquired nature of specificity and memory, hallmarks of a specific immune response.

[0005] Stimulation of an immune response is not limited to DNA plasmids or mRNA encoding for protein antigens. Non-coding bacterial DNA and oligonucleotides containing CpG motifs have also been shown to stimulate immunity (Yamamoto, S., *et al.*, *Microbiol. Immunol. 36*:983-997 (1992); Hacker, G., *et al.*, *Immunology 105*:245-251 (2002)).

[0006] DNA and RNA vaccines should elicit strong humoral and T-cell immune responses. However, in many cases the responses are not as strong as desired. This may be due to the ineffective targeting of antigen presenting cells (APC), such as macrophages and dendritic cells, by the DNA plasmids or RNA. A lack of targeting results in a significant transfection of other cells, such as myocytes, whose low class I major histocompatibility complex (MHC-1) levels and lack of costimulatory molecules such as B7 make them poor candidates for stimulation of antibodies or CTL. In effect, it has been shown that delivery of DNA to APC results in a rapid CTL induction and the production of higher avidity antibodies (Boyle, J.S., *et al., Proc. Nat. Acad. Sci. USA 94*:14626-14631(1997)). However, the quality of the immune response stimulated by these vaccines also depends on the recipient immune system's competence. Thus, compromised or weakened immune systems, such as those found in cancer patients and the elderly, might fail to mount an effective protective immune response without the help of one or more immune stimulants. In general, experimental DNA viral vaccines confer immunity on roughly half of the animals immunized, indicating the need for both APC targeting and immune stimulation.

[0007] Different procedures have been devised to avoid the limitations caused by the lack of targeting by DNA sequences (Lasic, D.D., *Liposomes in Gene Delivery,* CRC Boca Raton, 1997). In some cases, DNA plasmids have been enclosed in conventional liposomes to target macrophages. In other cases,. the DNA or RNA has been mixed with positively charged polymers to form complexes that are supposed to be taken up by APCs. In still others, the positively charged polymers have been conjugated to lipid chains, cholesterol or steroids, to facilitate the uptake of these nucleic acid complexes by cells via endocytosis, to avoid the lysosomal compartment and the concomitant nucleic acid degradation. Because classic liposomes do not significantly increase the intracellular delivery of nucleic acids, liposomes containing cationic lipids have been used instead. For example, enclosure of bacterial DNA or CpG oligonucleotides in liposomes containing cationic lipids has been shown to enhance their immunostimulatory properties (Yamamoto, T., *Microbiol. Immunol.* 38:831-836 (1994); Dow, S.W., *et al., J. Immunol.* 163:1552-1561 (1999; and Siders, W.F., *Mol. Ther.* 6:519-527 (2002)). Cationic lipids can form complexes with DNA that are able to transfect cells. However, cationic lipids have damaging effects on biological systems. For instance, they can induce platelet aggregation, hemolysis, cytotoxicity, and other damaging effects. This may limit their use to research only.

[0008] Therefore, there is a need for additional agents that either increase the amount of transfection or the degree of immune stimulation that occurs upon administration of a DNA or RNA vaccine.

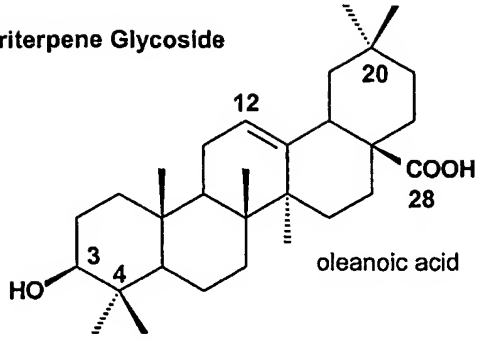
[0009] The inventions described herein address these needs by providing novel, effective compounds that i) facilitate the targeting and delivery of DNA or RNA to the APCs' cytosol, *i.e.* act as carriers, and/or ii) co-stimulate the immune system to produce an effective response, preferentially that of a Th1 type, *i.e.* to act as immune stimulants.

[0010] Saponins are glycosidic compounds that are produced as secondary metabolites. They are widely distributed among higher plants and in some marine invertebrates of the phylum Echinodermata (ApSimon *et al., Stud. Org. Chem. 17*:273-286 (1984)). Because of their antimicrobial activity, plant

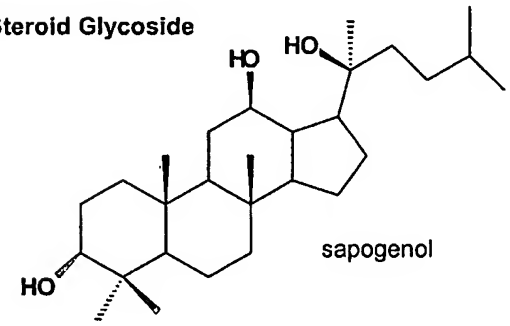
saponins are effective chemical defenses against microorganisms, particularly fungi (Price *et al., CRC Crit. Rev. Food Sci. Nutr. 26*:27-135 (1987)). Saponins are responsible for the toxic properties of many marine invertebrates (ApSimon *et al., Stud. Org. Chem. 17*:273-286 (1984)). The chemical structure of saponins imparts a wide range of pharmacological and biological activities, including some potent and efficacious immunological activity. In addition, members of this family of compounds have foaming properties (an identifying characteristic), surfactant properties (which are responsible for their hemolytic activity), cholesterol-binding, fungitoxic, molluscicidal, contraceptive, growth-retarding, expectorant, antiinflammatory, analgesic, antiviral, cardiovascular, enzyme-inhibitory, and antitumor activities (Hostettmann, K., *et al., Methods Plant Biochem. 7*:435-471(1991); Lacaille-Dubois, M.A. & Wagner, H., *Phytomedicine 2*:363-386 (1996); Price, K.R., *et al., CRC Crit. Rev. Food Sci. Nutr. 26*:27-135 (1987)).

[0011] Structurally, saponins consist of any aglycone (sapogenin) attached to one or more sugar chains. In some cases saponins may be acylated with organic acids such as acetic, malonic, angelic and others (Massiot, G. & Lavaud, C., *Stud. Nat. Prod. Chem. 15*:187-224(1995)) as part of their structure. These complex structures have molecular weights ranging from 600 to more than 2,000 daltons. The asymmetric distribution of their hydrophobic (aglycone) and hydrophilic (sugar) moieties confers an amphipathic character to these compounds which is largely responsible for their detergent-like properties. Consequently, saponins can interact with the cholesterol component of animal cell membranes to form pores that may lead to membrane destruction and cell death, such as the hemolysis of blood cells.

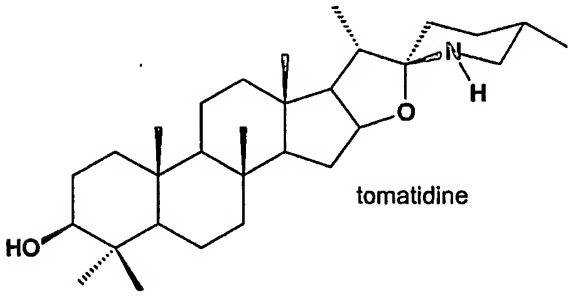
Triterpene Glycoside



Steroid Glycoside



Alkaloid Glycoside



[0012] Saponins can be classified according to their aglycone composition as shown above:

Triterpene glycosides

Steroid glycosides

Steroid alkaloid glycosides

[0013] The steroid alkaloid glycosides, or glycoalkaloids, share many physical and biological properties with steroid glycosides, but alkaloid glycosides are usually considered separately because their steroidal structure contains nitrogen. Frequently, the aglycones have methyl substituents that may be

oxidized to hydroxymethyl, aldehyde or carboxyl groups; these moieties may play a role in some of the saponins' biological activities. From extensive studies of saponins, it is apparent that the triterpene saponins are not only the most predominant in nature, but also those with the most interesting biological and pharmacological properties.

[0014] Saponins have one or more linear or branched sugar chains attached to the aglycone via a glycosidic ether or ester link. In some saponins, the presence of acylated sugars has also been detected. According to the number of sugar chains attached to the aglycone, the saponins can be monodesmosidic saponins (with a single sugar chain), or bidesmosidic saponins (with two sugar chains). In the monodesmosidic saponins, the sugar chain is typically attached by a glycosidic ether linkage at the C-3 of the aglycone. In addition to the C-3 linked sugar chain, bidesmosidic saponins have a second sugar chain bound at C-28 (triterpene saponins) or at C-26 (steroid saponins) by an ester linkage. Because of the typical lability of esters, bidesmosidic saponins are readily converted into their monodesmosidic forms by mild hydrolysis (Hostettmann, K., *et al., Methods Plant Biochem. 7*:435-471 (1991)).

[0015] Saponins from the bark of the *Quillaja saponaria* Molina tree (quillaja saponins) are chemically and immunologically well-characterized products (Dalsgaard, K. *Arch. Gesamte Virusforsch. 44*:243 (1974); Dalsgaard, K., *Acta Vet. Scand. 19 (Suppl. 69)*:1 (1978); Higuchi, R. *et al., Phytochemistry 26*:229 (1987); *ibid. 26*:2357 (1987); *ibid. 27:1168 (1988)*; Kensil, C. *et al., J. Immunol. 146*:431 (1991); Kensil *et al.*, U.S. Patent No. 5,057,540 (1991); Kensil *et al., Vaccines 92*:35 (1992); Bomford, R. *et al., Vaccine 10*:572 (1992); and Kensil, C. *et al.*, U.S. Patent No. 5,273,965 (1993)). From an aqueous extract of the bark of the South American tree, with *Quillaja saponaria* Molina, twenty-two peaks having saponin activity were separated by chromatographic techniques. The predominant purified saponins were identified as QS-7, QS-17, QS-18 and QS-21. QS-21 was later resolved into two additional peaks, each comprising a discrete compound, QA-21-V1 and QA-21-V2. See Kensil *et al.*, U.S. Patent No. 5,583,112 (1996).

[0016] These saponins are a family of closely related *O*-acylated triterpene glycoside structures. They have an aglycone triterpene (quillaic acid), with branched sugar chains attached to positions 3 and 28, and an aldehyde group in position 4. Quillaja saponins have an unusual fatty acid substituent (3,5-dihydroxy-6-methyloctanoic acid) as a diester on the fucose residue of the C-28 carbohydrate chain. This ester is hydrolyzed under mildly alkaline conditions or even at physiological pH over short periods of time to produce deacylated saponins, including DS-1 and DS-2 (Higuchi *et al., Phytochemistry* 26:229 (1987)); (Kensil *et al., Vaccines 92*:35-40 (1992)). More severe hydrolysis of these saponins using strong alkalinity (Higuchi *et al., Phytochemistry* 26:229 (1987)) or prolonged hydrolysis (Pillion, D.J., *et al., J. Pharm. Sci., 85*:518-524 (1996)) produces QH-957, the result of hydrolysis of the C-28 ester. The triterpenoid hydrolysis by-products have hydrophobic/hydrophilic properties differing from those of QS-21; these differences result in altered micellar and surfactant properties.

[0017] Some saponins have been shown to have different types of immune stimulating activities, including adjuvant activity. These activities have been reviewed previously (Shibata, S., *New Nat. Prod. Plant Pharmacol. Biol. Ther. Act., Proc. Int. Congr. 1st,* 177-198 (1977); Price, K.R., *et al., CRC Crit. Rev. Food Sci. Nutr. 26*:27-135 (1987); Schöpke, Th. and Hiller, K., *Pharmazie 45*:313-342 (1990); Lacaille-Dubois, M.A., *et al., Phytomedicine 2*:363-386 (1996); Press, J.B. *et al., Stud. Nat. Prod. Chem. 24*:131-174 (2000)). Immune adjuvants are compounds that, when administered to an individual, increase the immune response to an antigen in a test subject to which the antigen is administered, or enhance certain activities of cells from the immune system. Immune adjuvants modify or immunomodulate the cytokine network, up-regulating the humoral and cellular immune response. Humoral response elicits antibody formation. Cellular immune response involves the activation of T cell response, Thl or Th2, to mount this immune response. Th1 responses will elicit complement fixing antibodies and strong delayed-type hypersensitivity reactions associated with IL-2, IL-12, and γ-interferon. Induction of cytotoxic T lymphocytes (CTLs) response also

appears to be associated with a Th1 response. Th2 responses are associated with high levels of IgE, and the cytokines IL-4, IL-5, IL-6, and IL-10. The aldehyde-containing saponins such as those from quillaja induce a strong Th1 response. However, some of their analogs may induce a Th2 response.

[0018] Saponin adjuvants can target different cells, *i.e.*, macrophages, dendritic cells, hepatocytes, and others, by binding via their glycosyl residues to specific cell surface receptors. The saponins' triterpene or steroid moieties, by interacting with the cholesterol containing cell membrane lipid bilayer, allow the delivery of compounds complexed with the saponins directly to the cells' cytosol. Addition of a lipid side-chain to saponins results in a significant enhancement of this capacity. See Marciani, D.J., U.S. Patent No. 5,977,081 (1999). Saponins containing an aldehyde, by reacting with amino groups of receptor protein(s) present on certain T-cells and forming Schiff bases, stimulate Th1 immunity. Although saponins are effective adjuvants for proteins and carbohydrate antigens, they are not good carriers and/or stimulants of immunity when used in conjunction with DNA or RNA vaccines.

[0019] Novel cationic compounds have been synthesized by Ren *et al.* (*Tetrahedron Letts. 42*:1007-1010 (2001)) which contain trivalent galactosides that act to target specific cells for more effective transfection of DNA.

BRIEF SUMMARY OF THE INVENTION

[0020] The present invention is directed to novel saponin derivatives comprising:

(a) a saponin aglycone core, wherein the aglycone core is covalently linked to one or more oligosaccharide chains; and

(b) a positively charged cationic chain, wherein the cationic chain comprises (i) three or more carbon atoms; and (ii) one or more primary, secondary, or tertiary amine groups, or one or more guanidine groups, or any combination thereof; and wherein the cationic chain is covalently bound either to the aglycone core or to one or more oligosaccharide chains of the

derivative. The saponin derivative may further comprise (c) a naturally occurring or synthetic lipophilic chain, wherein the lipophilic chain comprises from 4 to 36 carbon atoms and optionally contains one or more oxyethylene groups.

[0021] The present invention is also directed to pharmaceutical and veterinary compositions comprising one or more of the saponin derivatives and one or more pharmaceutically acceptable diluents, carriers or excipients.

[0022] The present invention is further directed to a saponin derivative/polynucleotide complex comprising one or more of the saponin derivatives associated with a polynucleotide molecule. In this embodiment of the invention, the polynucleotide molecule is a non-coding bacterial DNA, or either DNA or RNA that at least partially encodes a peptide or polypeptide antigen. Useful antigens are peptide or polypeptide antigens associated with a pathogen such as a bacterium or virus that causes illness in a human or animal; or antigens associated with the presence of cancer in a human or animal.

[0023] The present invention is also directed to a saponin derivative/polynucleotide secondary complex comprising one or more saponin derivative/polynucleotide complexes described above in admixture or associated with one or more saponins selected from the group consisting of a native saponin, a semi-synthetic saponin derivative, and a synthetic saponin containing a triterpenoid aglycone core covalently linked to one or more oligosaccharide chains.

[0024] The present invention is further directed to pharmaceutical compositions comprising one or more saponin derivatives, a polynucleotide, and a pharmaceutically acceptable carrier or diluent; to a method of making the primary and secondary complexes described above; and to a method of making products produced by such methods.

[0025] The present invention is still further directed to a method of delivering a polynucleotide to cells of an animal in need thereof, comprising administration *in vivo* to an animal of a polynucleotide construct comprising a polynucleotide sequence encoding an immunogen, and one or more of the saponin derivatives of the invention. In this embodiment of the invention, the

polynucleotide sequence can be either DNA or RNA. If the polynucleotide sequence is DNA, the sequence may be operably linked to a promoter.

[0026] The present invention is also directed to a method of delivering a polynucleotide to cells of an animal in need thereof, comprising the steps of (a) forming a saponin derivative/polynucleotide complex, wherein the complex is comprised of one or more of the saponin derivatives of the invention associated with a polynucleotide sequence encoding an immunogen; and (b) administering the complex *in vitro* to the cells of the animal in an amount sufficient that uptake of said polynucleotide sequence into the cells of the animal occurs. In this embodiment of the invention, the polynucleotide sequence can be either DNA or RNA. If the polynucleotide sequence is DNA, the sequence may be operably linked to a promoter.

[0027] The present invention is further directed to a method of stimulating or generating an immune response in an animal in need thereof, comprising administering *in vivo* to the animal a polynucleotide sequence encoding an immunogen, and one or more of the saponin derivatives of the invention, in an amount sufficient that uptake of the polynucleotide sequence into cells of the animal occurs, and sufficient expression results, to stimulate or generate the immune response in the animal. In this embodiment of the invention, the polynucleotide sequence can be a DNA sequence linked to a promoter.

[0028] The present invention is also directed to a method of stimulating or generating an immune response in an animal in need thereof, comprising administering *in vivo* to the animal a noncoding bacterial DNA polynucleotide and one or more of the saponin derivatives of the invention, to stimulate or generate the immune response in the animal. The method can further comprise administering *in vivo* to the animal a polypeptide antigen, or a polynucleotide sequence encoding an immunogen.

[0029] The present invention is also directed to a method of stimulating or generating an immune response in an animal in need thereof, comprising the steps of (a) introducing into the cells of the animal a polynucleotide sequence encoding an immunogen, and one or more of the saponin derivatives of the invention; and (b) introducing the cells into the animal, wherein sufficient

expression of the immunogen occurs in the cells and an immune response is stimulated or generated in the animal. In this embodiment of the invention, the polynucleotide sequence can be a DNA sequence that is operably linked to a promoter.

[0030] The present invention is also directed to a method of generating a detectable immune response in an animal in need thereof, comprising administering *in vivo* to the cells of an animal a polynucleotide sequence encoding an immunogen, and one or more of the saponin derivatives of the invention, in an amount sufficient that uptake of the polynucleotide sequence into the cells of the animal occurs, and sufficient expression results, to generate the detectable immune response. In this embodiment of the invention, the polynucleotide sequence can be a DNA sequence that is operably linked to a promoter.

[0031] The present invention is further directed to a method of generating a detectable immune response in an animal in need thereof, comprising the steps of (a) introducing into the cells of the animal a polynucleotide sequence encoding an immunogen, and one or more of the saponin derivatives of the invention; and (b) introducing the polynucleotide sequence into the cells into the animal, wherein sufficient expression of the immunogen occurs in the cells and a detectable immune response is generated. In this embodiment of the invention, the polynucleotide sequence can be a DNA sequence that is operably linked to a promoter.

BRIEF DESCRIPTION OF THE FIGURES

[0032] FIG. 1 illustrates the effects of DMPS (3-dimethylamino-1-propylamino-DS-saponin derivative (compound III in Scheme 1a)) on the immune response of individual female Balb/c mice to OVA DNA.

[0033] FIG. 2 illustrates the effects of DMPS on the immune response of female Balb/c mice to OVA DNA, expressed as average values of absorbance at 450 nm.

[0034] FIG. 3 illustrates the effects of DMPS (GPI-0330) on the IgG1 and IgG2a response to OVA DNA vaccination.

[0035] FIG. 4. illustrates the effects of the polyethylenimine quillaja saponin derivative of Example 5d (GPI-0332) on the IgG1 and IgG2a response to OVA DNA vaccination.

DETAILED DESCRIPTION OF THE INVENTION

[0036] The present invention is directed to novel saponin derivatives comprising:

(a) an aglycone core substituted with one or more oligosaccharide chains; and

(b) a positively charged cationic chain comprising (i) three or more carbon atoms, and (ii) one or more primary, secondary, or tertiary amine groups, or one or more guanidine groups, or any combination thereof. The novel saponin derivatives may optionally include a naturally occurring or synthetic lipophilic chain covalently attached to either the aglycone core or to one or more of the oligosaccharide chains.

[0037] Appropriate saponins include triterpene glycosides, steroid glycosides, and steroid alkaloid glycosides, with triterpene glycosides the most preferred saponins. Thus a preferred aglycone core is a triterpenoid aglycone core.

[0038] One or more oligosaccharide chains may be covalently liked to the aglycone core. If the aglycone core is a triterpene nucleus, there are preferably one or two oligosaccharide chains linked at positions 3 and/or 28 of the triterpene nucleus. The attached oligosaccharide chains are capable of binding to carbohydrate receptors on the cells' surface, preferentially of APCs, such as macrophages and dendritic cells.

[0039] The saponin derivative may have an aldehyde or ketone group, preferably an aldehyde group, in its aglycone or its oligosaccharide chains that is capable of forming an imine or Schiff base with an amino group. The formation of an imine or Schiff base with certain cell surface receptors, preferentially on an APC, provides a co-stimulatory signal needed for

stimulation of an immune response, preferentially of type Th1. If the aldehyde or ketone group is attached to the aglycone core, the aldehyde or ketone group will be attached preferably at position 4 of the core.

[0040] Quillaja, Gypsophila and Saponaria are saponins useful in the present invention, all having triterpene aglycones with an aldehyde group linked or attached to position 4, branched oligosaccharides linked by an ester bond in position 28, and a 3-O-glucuronic acid (3-O-glcA) that in Quillaja and Gypsophila is linked to branched oligosaccharides. Saponins from Q. saponaria and S. jenisseenis include acyl moieties, whereas saponin from Gypsophila, Saponaria, and Acanthophyllum do not include acyl moieties. Each of these non-acylated or de-acylated saponins is useful in the present invention. Saponins without aldehyde groups, such as soyasaponins, camellidin, and dubioside F, are also useful in the present invention.

[0041] Other triterpene saponins are suitable for use in the present invention and include, for example, the bidesmosidic saponin, squarroside A, isolated from *Acanthophyllum squarrosum*; the saponin lucyoside P; and two acylated saponins isolated from *Silene jenisseensis* Willd. See, for example, U.S. Patent No. 6,080,725.

[0042] Attached to the saponin derivative is a positively charged cationic chain, which is covalently bound to either the aglycone core or to a sugar residue of an oligosaccharide chain of the saponin derivative. The cationic chain can have a molecular weight of 100 to 100,000 daltons and may have one or more positively charged cationic groups. For purposes of the present invention, the cationic group must possess a positive charge under particular environmental or physiological conditions. Thus, amine groups are considered to be cationic since amine groups are protonated under a variety of environmental and physiological conditions.

[0043] In the present invention, the cationic chain can be any cationic amine capable of being linked to the aglycone core or to a sugar residue. Thus the cationic chain must contain at least one of the following chemical groups: a carboxyl group, a primary or secondary amine group, a thiol group, a hydroxyl group, or a chemical group capable of being activated to form a covalent bond

to the aglycone and/or sugar moieties of a saponin. See Behr *et al.* (*Proc. Natl. Acad. Sci. 86*:6982-6986 (1989)) and Wheeler (U.S. Patent No. 5,861,397) for examples of cationic chains that are suitable for use in the present invention.

[0044] Preferably, the cationic chain comprises (i) a minimum of three (3) carbon atoms and (ii) contains one or more primary, secondary, or tertiary amine groups, or one or more guanidine groups, or any combination thereof. The cationic chain can be a linear or branched aliphatic chain. Examples of cationic chain aliphatic groups include straight-chained or branched, saturated or unsaturated aliphatic groups having about 3 to about 24 carbon atoms, preferably 3 to 20 carbon atoms, more preferably 3 to 16 carbon atoms, and most preferably 6 to 12 carbon atoms. Examples of useful aliphatic groups include pentyl, hexyl, heptyl, octyl, nonyl, decyl, dodecyl, and hexadecyl. Examples of preferred aliphatic amine cationic chains include 3-dimethylamino-1-propylamino, 3-trimethylamino-1-propylamino, 5-dimethyl-1-pentylamino; polyamine chains having 10-16 atoms, such as spermine and spermidine; aliphatic chains containing one or more pyridinium, pyrimidinium, or imidazolinium groups; and choline.

[0045] Additional examples of preferred cationinc chains include linear and branched polyethylenimines, glucosamine, and mannosamine.

[0046] The positively charged cationic chain can also be an oligosaccharide or a polysaccharide; a protein, such as a histone or protamine; or a synthetic or natural oligopeptide or polypeptide with a series of basic amino acids, *i.e.* lysine and arginine, such as a polylysine chain. The positively charged cationic chain can also be a polypeptide that is cationic or has been subsequently modified by the introduction of amino groups or similar cationic basic groups that are capable of forming a complex with DNA or RNA.

[0047] Proteins and polypeptides can be modified by introducing cationic groups using one of the following methods: *i*) introducing an amine group at the carboxyl group of a protein or polypeptide by reaction with a diamine (*e.g.*, ethylenediamine, Jeffamine EDR-148) using carbodiimide mediated coupling, with active ester intermediates such as NHS esters, or with agents

such as N,N'-carbodiimidazole; *ii*) creating a carboxylate group from a hydroxyl group by reaction with choloroacetic acid. The new carboxyl group can be modified by reaction with a diamine as previously described; *iii*) modifying sulfhydryl groups with N-(β-iodoethyl)trifluoroacetamide to yield an intermediate that undergoes spontaneous deblocking, yielding an aminoethyl derivative linked via a thioether; *iv*)modifying sulfhydryls with ethylenimine or with 2-bromoethylamine to yield an aminoethyl derivative (see Hermanson, G.T., *Bioconjugate Techniques*, Academic Press, New York, 1996); *v*) converting a sulfhydryl group to a basic derivative, 4-thialaminine, by alkylation with (2-bromoethyl)trimethylammonium; *vi*) treating a protein with O-methylisourea at alkaline pH to convert primary amino groups to the more basic guanidinium groups, *i.e.*, changing the lysyl residues to homoarginine derivatives.

[0048] The saponin derivative, in addition to the positively cationic charged chain, may optionally have a lipophilic chain. This lipophilic chain may be linked to the aglycone core or to a sugar residue of an oligosaccharide chain of the saponin derivative. The lipophilic chain comprises 4 to 36 carbon atoms, preferably 10 to 14 carbon atoms, and most preferably 12 carbon atoms, and may be linear or branched, and saturated or unsaturated and may optionally contain one or more oxyethylene groups. By way of example, useful lipophilic chains include fatty acids, terpenes, polyethylene glycols, and linear or branched lipid chains. Additional useful lipophilic chains include those described in U.S. Patent No. 6,262,029 ("Chemically Modified Saponins and the Use Thereof as Adjuvants"), at columns 7 to 11. Lipophilic chains suitable for use in the present invention do not contain cationic groups such as primary, secondary or tertiary amine groups.

[0049] Useful fatty acid lipophilic chains include C_6-C_{24} fatty acids, preferably C_7-C_{18} fatty acids. Examples of preferred useful fatty acids include saturated fatty acids such as lauric, myristic, palmitic, stearic, arachidic, behenic, and lignoceric acids; and unsaturated fatty acids, such as palmitoleic, oleic, linoleic, linolenic and arachidonic acids.

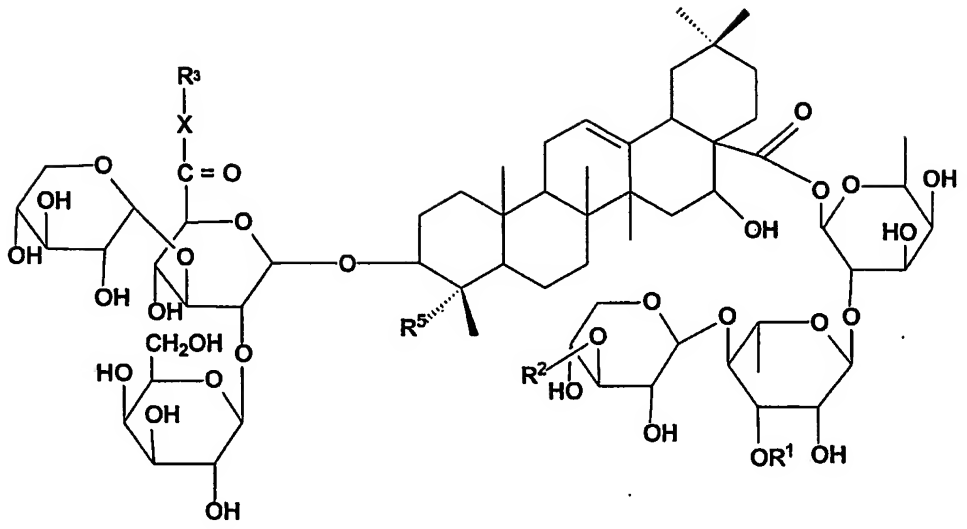
[0050] Useful terpenoids include retinol, retinal, bisabolol, citral, citronellal, citronellol and linalool.

[0051] Useful polyethylene glycols have the formula $H-(O-CH_2-CH_2)_nOH$, where *n*, the number of ethylene oxide units, is from 4 to 14. Examples of useful polyethylene glycols include PEG 200 (*n*=4), PEG 400 (*n*=8-9), and PEG 600 (*n*=12-14). Useful polyethylene glycol fatty alcohol ethers include those wherein the ethylene oxide units (*n*) are between 1 to 8, and the alkyl group is from C_6 to C_{18}.

[0052] In a first preferred embodiment of this invention, the positively charged cationic chain of the saponin derivative is covalently linked to one of the glycosyl residues of the saponin, preferentially to a carboxylic group, such as that present on glucuronic and galacturonic acid residues.

[0053] For compounds of this embodiment, the cationic chain can be linked to a carboxyl group via one of their primary amino groups using the carbodiimide reaction in the presence of N-hydroxysuccinimide (NHS) or their water-soluble analogs. See Schemes 1a-1c and the syntheses described in Example 1 below. For small cationic chains (C3 to C18) carrying 2 to or more amino groups, the reaction is carried out in the presence of an excess of the cationic chain, to avoid the incorporation of multiple saponin groups to the chain. For large cationic chains, (polylysine, protamines and others), the number of saponin residues per chain can be adjusted by increasing or decreasing the relative proportions of saponin and cationic chain. In both cases, the resulting compounds would have saponin residues that might or might not carry aldehyde groups. These derivatives would not carry a lipophilic side-chain.

[0054] Thus, saponin derivatives of this embodiment include a compound of Formula I:



(I)

or a pharmaceutically acceptable salt thereof; wherein

R^1 is glucose or hydrogen;

R^2 is apiose or xylose, preferably apiose;

X is –NH;

R^3 is C_4-C_{30} alkyl or C_4-C_{30} alkenyl, either of which is optionally substituted by one or more $-NH_2$, $-NHR^4$, $-N(R^4)_2$, $-NH_3^+$, $-(NH_2R^4)^+$, $-N(R^4)_3^+$, or $-NH((R^4)_2N)C(=NR^4)$, where R^4 is hydrogen or lower alkyl; and either of which is optionally interrupted by one or more NH, NH_2^+, S, O, C=O, or NR^4 groups; or

R^3 is an oligosaccharide, polysaccharide, or protein; and

R^5 is CH=O, CH_3, or CH_2OH.

[0055] In a preferred aspect, R^3 is selected from the group consisting of a C_4-C_{30} straight or branched chain alkyl group, and a C_4-C_{30} straight or branched chain alkenyl group; either of which is optionally substituted by one or more $-NH_2$, $-NHR^4$, $-N(R^4)_2$, or $-NH(H_2N)C(=NH)-$, where R^4 is hydrogen or a lower alkyl, preferably methyl, and either of which is optionally interrupted by one or more NH groups. Preferred R^3 groups in this aspect include aliphatic amines and polyamines.

[0056] In a second preferred aspect, R^3 is an oligosaccharide, polysaccharide, or protein. Preferred oligosaccharides and polysaccharides include those

composed mostly of amino sugars such as glucosamine and mannosamine, or chemically aminated sugars.

[0057] Non-limiting examples of saponin derivatives of this first embodiment include compound III of Scheme 1a and compound VIII of Scheme 1c.

[0058] In a second preferred embodiment of the invention, the saponin derivatives have a positively charged cationic chain attached to the aldehyde group on the aglycone nucleus of the saponin.

[0059] Thus, compounds of this second embodiment include compounds of Formula I wherein:

X is -O-; R^3 is H;

R^5 is -CHNHR^6, wherein R^6 is C_4-C_{30} alkyl or C_4-C_{30} alkenyl, either of which is optionally substituted by one or more -NH_2, -NHR^4, -$N(R^4)_2$, -NH_3^+, -$(NH_2R^4)^+$, -$N(R^4)_3^+$, or -$NH((R^4)_2N)C(=NR^4)$, where R^4 is hydrogen or lower alkyl; and either of which is optionally interrupted by one or more NH or NH_2^+ groups; or R^6 is an oligosaccharide or polysaccharide, preferably an oligosaccharide or polysaccharide composed of aminated sugars, or R^6 is a protein; and

R^1 and R^2 have the same definitions are indicated above for compounds of the first embodiment. Examples of compounds in this embodiment are presented in Scheme 2.

[0060] For compounds in this second embodiment, the cationic chain can be linked to the aldehyde using reductive amination in the presence of Na cyanoborohydride or Na borohydride. See, for example, the synthesis outlined in Scheme 2a and in Example 2 below. As for compounds of the first embodiment (Formula I), the number of saponin residues per cationic chain can be selected by adjusting the relative proportions of the glycoside or saponin and the cationic polymer. Because the aldehyde group will be used during the reaction with the primary amine, the resultant saponin derivative does not have the capacity to co-stimulate T-cells. Thus, compounds of this second embodiment of the invention can be used in combination with natural saponins, semi-synthetic or synthetic saponin derivatives carrying aldehyde groups. Formation of micelles between the cationic chain-saponin

derivative/polynucleotide complex and the aldehyde-carrying saponin will deliver co-stimulatory signals to the T-cells.

[0061] Non-limiting examples of compounds of this second embodiment include compound X of Scheme 2a.

[0062] In a third embodiment of the invention, the saponin derivatives have structures similar to that described for compounds of the first two embodiments, but a lipophilic chain is attached to the saponin residues of these derivatives. For those compounds of this embodiment in which the cationic chain is linked to a sugar residue, the lipophilic chain can be added at the aldehyde group of the aglycone nucleus by reductive amination with an alkyl monoamine, such as dodecylamine. See, for example, Scheme 3a and Example 3 below. For those compounds in which the positively charged cationic chain attached to the aldehyde group of the aglycone nucleus, the lipophilic chain can be added to a sugar residue, such as glucuronic acid, by reacting with an alkyl monoamine in the presence of carbodiimide and NHS. In both cases, the corresponding derivatives lack or have a limited number of aldehyde residues. If co-stimulation is required, then the nucleic acid complexes formed with these derivatives must be used in combination with aldehyde-carrying native saponins or their semi-synthetic derivatives, such as GPI-0100.

[0063] Non-limiting examples of compounds of this embodiment include compound XIII of Scheme 3a below.

[0064] In a fourth embodiment of the invention, the saponin derivatives have a lipophilic chain which is attached to a sugar residue of the oligosaccharide chain of the saponin, preferentially to the carboxyl group of a glucuronic or galacturonic acid. Scheme 4a outlines the synthetic steps for preparing a compound of this embodiment.

[0065] Non-limiting examples of compounds of this embodiment include compound XIV in Scheme 4a below.

[0066] In a fifth embodiment of the invention, the cationic chain of the saponin derivatives is a protein or a polymer. Compounds of this embodiment include conjugates between a saponin (such as desacylated quillaja saponin,

gypsophylla saponin, and other similar glycosides or saponins) and *i*) a protein such as protamine or histone, or *ii*) a polymer such as polylysine, polyethylenimine, polyglucosamine or chitosan. The polymers are linked to the glycoside or saponin moiety by either the carboxyl or aldehyde groups. Schemes 5a-5c outline the synthetic steps required for preparation of three compounds of this fifth embodiment.

[0067] Non-limiting examples of compounds of this fifth embodiment include compound XV in Scheme 5a and compound XVIII in Scheme 5b below.

Preparation of the Saponin Derivatives

[0068] Cationic saponin derivatives of the present invention can be synthesized from saponin starting materials using conventional synthetic protocols known to those of ordinary skill in the art. See, for example, U.S. Patent No. 6,080,725, for a description of synthetic protocols used in the preparation of desacylsaponin starting materials.

[0069] Schemes 1a-5c and Examples 1-5 herein provide synthetic protocols for the preparation of specific cationic saponin derivatives of the invention. One of ordinary skill in the art will know how to use these synthetic protocols, and adapt them when necessary, to prepare additional saponin derivatives falling within the scope of the invention.

Use of the Saponin Derivatives with DNA and RNA polynucleotides

[0070] The saponin derivatives of the present invention can be combined with DNA or RNA polynucleotides and used to enhance the immune response of an animal or to stimulate or generate an immune response in an animal. For example, the saponin derivatives can be used with coding or noncoding bacterial DNA, plasmid DNA, polynucleotides or CpG oligonucleotides to stimulate a non-specific innate immune response in an animal. The term "noncoding bacterial DNA," as used herein, refers to DNA of bacterial origin that does not encode a known antigen. See, for example, Hacker, G., *et al.*, *Immunology 105*:245-251 (2002); Siders, W.F., *Mol. Ther.* 6:519-527 (2002); and Klinman, D.M., *et al.*, *Proc. Natl. Acad. WSci. USA 93*:2879-2883 (1996).

Noncoding bacterial DNA polynucleotides can be in linear, circular (e.g., a plasmid), or branched form; and in double-stranded or single-stranded form. Bacterial double-stranded DNA plasmids are preferred for use with the saponin derivatives of the invention.

[0071] CpG oligonucleotides can also be used with saponin derivatives of the invention to stimulate a non-specific innate immune response in an animal. The term "CpG oligonucleotide" refers to DNA polynucleotides of about 20 to about 25 nucleotides or less, which contain one or more CpG dinucleotide motifs. CpG oligonucleotides can be single-stranded or double-stranded. Double-stranded DNA CpG oligonucleotides of about 20 base pairs are preferred.

[0072] In some aspects of the present invention, the saponin derivatives described herein are administered to an animal in conjunction with a bacterial DNA polynucleotide or a CpG oligonucleotide. The saponin derivatives can associate with the polynucleotides or oligonucleotides (via salt linkages) to form complexes that are fairly stable under physiological conditions. These complexes should be reversible and able to dissociate in the presence of pH changes, or some agents, such as certain proteins or salts, to yield free polynucleotide or oligonucleotide.

[0073] Bacterial DNA/saponin derivative complexes and CpG oligonucleotide/saponin derivative complexes may also be administered with an antigen polypeptide or with a coding DNA or RNA vaccine, as described below, to stimulate or generate a specific immunity in an animal. The polypeptide antigen or DNA or RNA vaccine is preferably administered in combination with the bacterial DNA/saponin derivative complexes or in combination with the CpG oligonucleotide/saponin derivative complexes. Thus, for example, in some aspects of the invention, the polypeptide antigen is included in, or forms a part of, the bacterial DNA/saponin derivative complex that is administered to an animal.

[0074] The saponin derivatives of the present invention can also be utilized to enhance the immune response of an animal against specific antigens produced by the use of nucleic acid vaccines. Typical vaccines using this approach are

viral vaccines, such as influenza, herpes, cytomegalovirus, HIV-1, HTLV-1, FIV, cancer vaccines, and parasitic vaccines. DNA vaccines are also currently being developed for prevention and treatment of a number of infectious diseases. Boyer, J., *et al., Nat. Med. 3*:526-532 (1997); reviewed in Spier, R., *Vaccine 14*:1285-1288 (1996).

[0075] In a DNA or RNA vaccine, a polynucleotide operatively coding for an immunogenic polypeptide in a pharmaceutically acceptable administrable carrier is administered to the cells of an animal suffering from cancer or pathogenic infection, wherein the polynucleotide is incorporated into the cells and an amount of an immunogenic polypeptide is produced capable of stimulating a preventive or therapeutically effective immune response.

[0076] The polynucleotide material delivered to the cells can take any number of forms. It may contain the entire sequence or only a fragment of an immunogenic polypeptide gene. It may also contain sequences coding for other polypeptide sequences. It may additionally contain elements involved in regulating gene expression (*e.g.*, promoter, enhancer, 5′ or 3′ UTRs, transcription terminators, and the like). The polynucleotide may also comprise an immunostimulatory sequence that would enhance the immunogenicity of a given gene product, and/or it may comprise sequences that would enhance the delivery of the polynucleotide, such as by increasing cellular and/or nuclear uptake. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman *et al., Proc. Nat. Acad. Sci. (USA) 84*:2150-2154 (1987), which is hereby incorporated by reference.

[0077] The polynucleotide material delivered to the cells can also be antisense DNA or antisense RNA. Thus, in the present invention, the saponin derivatives described herein can be utilized to deliver antisense DNA or RNA into target cells. Cell targeting depends on the sugars attached to the saponin aglycone core. Thus, it is possible to modify or replace one or more oligosaccharide chains in the saponin derivatives chosen for use with the antisense DNA or RNA with other sugar residues that will target the saponin derivative/antisense polynucleotide complex to particular types of cells.

[0078] In a preferred aspect of the present invention, the saponin derivatives described herein are administered to an animal in conjunction with a DNA or RNA vaccine comprising a polynucleotide, *i.e.*, DNA or RNA, that encodes an antigen. The saponin derivatives associate with the polynucleotide and facilitate targeting of the polynucleotide to APCs of the animal, such that the polynucleotide is incorporated into the cells of the animal, a therapeutically effective amount of the encoded antigen is produced, and an effective immune response is produced in the animal.

[0079] The saponin derivatives administered with the nucleic acid vaccine have the capacity to form complexes with the DNA or RNA polynucleotides of the vaccine (via salt linkages) that are fairly stable under physiological conditions. These complexes should be reversible and able to dissociate in the presence of pH changes, or some agents, such as certain proteins or salts, to yield free DNA or RNA. The strength of the association between the DNA/RNA and the saponin derivative may be gauged by adjusting the length of the cationic chain attached to the saponin moiety and the nature and/or density of its basic groups.

[0080] The DNA or RNA complexes formed with the saponin derivatives (*i.e.*, the saponin derivative/polynucleotide complex) disclosed here may also interact with *i*) native saponins, such as those from quillaja, gypsophila or similar ones; *ii*) semi-synthetic saponin derivatives such as GPI-0100 and similar ones; or *iii*) synthetic glycosides containing a triterpenoid aglycone linked to one or more carbohydrate chains. The aglycone core may or may not carry an aldehyde or ketone group. These interactions occur between the saponin moieties of the present invention and the natural or semi-synthetic saponin derivatives to form mixed micelles or similar aggregates in the presence or absence of DNA or RNA. These micelles or aggregates should also occur in the presence of non-ionic detergents, such as polyoxyethylene fatty acid esters, polyoxyethylene sorbitan fatty acid esters, and others, forming mixed micelles containing the non-ionic detergent. The natural glycosides or saponins, their semi-synthetic derivatives and synthetic products capable of interacting with the glycoside or saponin moieties of the present

invention, should preferentially have an aldehyde or ketone group to provide a co-stimulatory signal to an APC, and a lipophilic side chain capable of interacting with the cell membrane to facilitate the delivery of the nucleic acid to the cytosol.

[0081] The DNA or RNA complex formed with the modified saponins of the present invention should bind to cell receptors for carbohydrates, preferentially on APCs, by the saponins' carbohydrate residues. Alternatively, after forming a saponin derivative/polynucleotide complex, the modified saponins of the present invention would associate with either natural, semi-synthetic or synthetic derivatives of saponins, preferably derivative of triterpenoid saponins, preferentially carrying an aldehyde, to form micelles or similarly aggregated structures. These aggregates would then bind to the cell-surface receptors for the saponins' carbohydrate residues, mediate the delivery of DNA of RNA to the cell's cytosol compartment, and if they contain an aldehyde group, co-stimulate the T-cells. The presence of a co-stimulatory signal like the aldehyde group, may help avoid the problem of "anergy". This anergy or immune tolerance is caused by the interaction between the T Cell Receptor (TCR) and the APC's MHC-1/peptide complex, but without the concomitant co-stimulation by B7-1. In the present invention, the modified saponin DNA carrier provides such a co-stimulatory signal via aldehyde groups present on the carrier itself or in other glycosides associated with the carrier.

[0082] The methods of the invention may be carried out by direct delivery to the mucosal membranes or by direct injection of the saponin derivative/polynucleotide complex into the animal *in vivo*, or by *in vitro* transfection of some of the animal cells which are then re-introduced into the animal's body.

[0083] Thus, the present invention provides a method of immunizing an animal, wherein a preparation of a saponin derivative/polynucleotide complex is obtained that comprises one or more saponin derivatives of the invention and a polynucleotide construct comprising a polynucleotide coding for an antigenic peptide. The saponin derivative/polynucleotide complex is then

introduced into an animal, whereby the polynucleotide construct is incorporated into an APC (a monocyte, a macrophage, a dendritic cell, or another cell), where an antigenic translation product of the polynucleotide is formed, and the product is processed and presented by the cell in the context of the major histocompatibility complex, thereby eliciting an immune response against the antigen. Again, the polynucleotide is DNA or RNA, but preferably mRNA. If the polynucleotide is DNA, the gene for an antigen ("immunogen") is present on the polynucleotide. If the polynucleotide is mRNA, the mRNA, when translated, produces the antigen.

[0084] In an alternative embodiment, the present invention also provides a method of immunizing an animal, wherein one or more cells are removed from an animal and the cells are transfected *in vitro* with a saponin derivative/polynucleotide complex that comprises one or more saponin derivatives of the invention and a polynucleotide construct comprising a polynucleotide coding for an antigenic peptide. The polynucleotide construct of the complex is incorporated into the cells and an antigenic translation product of the polynucleotide is formed. After transfection, the cells, now expressing the antigen, are reinjected into the animal where the immune system can respond to the (now) endogenous antigen and an immune response against the immunogen is elicited. In this embodiment of the invention, the cells to be transfected with the saponin/polynucleotide complex are preferably lymphoid cells, more preferably APC's, which have been removed from an animal.

[0085] If cells from the animal are to be transfected *in vitro* in practice of the invention, the source of the cells can be peripheral blood cells, which can be rapidly isolated from whole blood to provide a source of cells containing both class I and class II MHC proteins. These cells can be further fractionated into B cells, helper T cells, cytotoxic T cells or macrophage/monocyte cells if desired (APC's). Bone marrow cells can provide a source of less differentiated lymphoid cells. In all cases the cell will be transfected *in vitro* either with DNA containing a gene for the antigen or by the appropriate capped and polyadenylated mRNA transcribed from that gene or a circular

RNA, chemically modified RNA, or an RNA which does not require 5' capping. The choice of the transfecting nucleotide may depend on the duration of expression desired. For vaccination purposes, a reversible expression of the immunogenic peptide, as occurs on mRNA transfection, is preferred. Transfected cells are injected into the animal and the expressed proteins will be processed and presented to the immune system by the normal cellular pathways.

[0086] As used herein, the term "antigen" means a substance that has the ability to induce a specific immune response. For purposes of the present invention, the term "antigen" is used interchangeably with "immunogen".

[0087] Any appropriate antigen which is a candidate for an immune response, whether humoral or cellular, can be used in the invention. In any of the embodiments of the invention, the immunogenic product may be secreted by the cells, or it may be presented by a cell of the animal in the context of the major histocompatibility antigens, thereby eliciting an immune response against the immunogen. The invention may be practiced using non-dividing, differentiated APCs from the vertebrates, such as lymphocytes obtained from a blood sample.

[0088] Since the immune systems of all vertebrates operate similarly, the applications described can be implemented in all vertebrate systems, comprising mammalian and avian species, as well as fish. Any vertebrate that may experience the beneficial effects of the compositions and applications of the present invention is within the scope of subjects that may be treated.

[0089] The subjects are preferably mammals. The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not limited to, primate mammals such as human, apes, monkeys, orangutans, and chimpanzees; canine mammals such as dogs and wolves; feline mammals such as cats, lions, and tigers; equine mammals such as horses, donkeys, deer, zebras, and giraffes; and common domesticated mammals such as cattle, sheep, and pigs. Preferably, the mammal is a human subject.

[0090] In a preferred aspect of the invention, the polynucleotide construct of the nucleic acid vaccine to be used with the saponin derivatives of the present invention comprises at least one polynucleotide (e.g., DNA, RNA, ribozyme, phosphorothioate, or other modified nucleic acid) encoding one or more antigens. The polynucleotide can be provided in linear, circular (e.g. plasmid), or branched form; and double-stranded or single-stranded form. The polynucleotide can involve a conventional phosphodiester bond or a non-conventional bond (e.g., an amide bond as in peptide nucleic acid (PNA)). The choice of polynucleotide encoding an antigen will depend on the desired kinetics and duration of expression. When long term delivery of the polynucleotide construct is desired, the preferred polynucleotide is DNA. Alternatively, when short term delivery is desired, the preferred polynucleotide is mRNA. RNA will be rapidly translated into polypeptide, but will be degraded by the target cell more quickly than DNA. In general, because of the greater resistance of circular DNA molecules to nucleases, circular DNA molecules will persist longer than single-stranded polynucleotides, and they will be less likely to cause insertional mutation by integrating into the target genome.

[0091] In a preferred embodiment, the polynucleotide sequence encoding one or more antigens is RNA. Most preferably, the RNA is messenger RNA (mRNA). A viral alphavector, a non-infectious vector useful for administering RNA, may be used to introduce RNA into animal cells. Methods for the *in vivo* introduction of alphaviral vectors to mammalian tissues are described in Altman-Hamamdzic, S., *et al.*, *Gene Therapy 4*: 815-822 (1997), which is herein incorporated by reference.

[0092] In another embodiment of the invention, the polynucleotide sequence encoding one or more antigens is DNA. In a DNA construct, a promoter is preferably operably linked to the polynucleotide encoding an antigen. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, can be included in the polynucleotide construct to direct cell-specific transcription of the DNA.

[0093] An operable linkage is a linkage in which a polynucleotide sequence encoding an antigen is connected to one or more regulatory sequence in such a way as to place expression of the antigen sequence under the influence or control of the regulatory sequence(s). Two DNA sequences (such as a coding sequence and a promoter region sequence linked to the 5' end of the coding sequence) are operably linked if induction of promoter function results in the transcription of mRNA encoding the desired polypeptide and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the polypeptide, antisense RNA, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably linked to a DNA sequence if the promoter was capable of effecting transcription of that DNA sequence.

[0094] Preferably, the polynucleotide construct is a circular or linearized plasmid containing non-infectious, nonintegrating nucleotide sequence. A linearized plasmid is a plasmid that was previously circular but has been linearized, for example, by digestion with a restriction endonuclease. The polynucleotide sequence encoding an antigen may comprise a sequence which directs the secretion of the antigenic polypeptide.

[0095] "Noninfectious" means that the polynucleotide construct does not infect mammalian cells. Thus, the polynucleotide construct can contain functional sequences from non-mammalian (e.g., viral or bacterial) species, but the construct does not contain functional non-mammalian nucleotide sequences that facilitate infection of the construct into mammalian cells.

[0096] "Nonintegrating" means that the polynucleotide construct does not integrate into the genome of mammalian cells. The construct can be a non-replicating DNA sequence, or specific replicating sequences genetically engineered to lack the ability to integrate into the genome. The polynucleotide construct does not contain functional sequences that facilitate integration of the antigen-encoding polynucleotide sequence into the genome of mammalian cells.

[0097] The polynucleotide construct is assembled out of components where different selectable genes, origins, promoters, introns, 5′ untranslated (UT) sequence, terminators, polyadenylation signals, 3′ UT sequence, and leader peptides, etc. are put together to make the desired vector. The precise nature of the regulatory regions needed for gene expression can vary between species or cell types, but shall in general include, as necessary, 5′ non-transcribing and 5′ non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, CAAT sequence, and the like, with those elements necessary for the promoter sequence being provided by the promoters of the invention. Such transcriptional control sequences can also include enhancer sequences or upstream activator sequences, as desired.

[0098] The polynucleotide construct can be an expression vector. A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the polypeptide coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript, as well as additional elements that include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Suitable expression vectors for use in practicing the present invention include, for example, vectors such as PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), VR1012, VR1055, and pcDNA3 (Invitrogen, San Diego, CA). All forms of DNA, whether replicating or non-replicating, which do not become integrated into the genome, and which are expressible, can be used in the methods contemplated by the invention.

[0100] The vector containing the DNA sequence (or the corresponding RNA sequence) which can be used in accordance with the invention can be a eukaryotic expression vector. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman *et al., Proc. Nat. Acad. Sci. (USA) 84*:2150-2154 (1987), which is herein incorporated by reference.

[0101] The present invention also encompasses the use of DNA coding for a polypeptide and for a polymerase for transcribing the DNA, and wherein the DNA includes recognition sites for the polymerase. The initial quantity of polymerase is provided by including mRNA coding therefor in the preparation, which mRNA is translated by the cell. The mRNA preferably is provided with means for retarding its degradation in the cell. This can include capping the mRNA, circularizing the mRNA, or chemically blocking the 5' end of the mRNA. The DNA used in the invention may be in the form of linear DNA or may be a plasmid. Episomal DNA is also contemplated. One preferred polymerase is phage T7 RNA polymerase and a preferred recognition site is a T7 RNA polymerase promoter.

[0102] For the methods of the present invention, a single polynucleotide construct containing more than one polynucleotide sequence encoding one or more molecules may be used according to the invention. Alternatively, more than one polynucleotide construct each containing polynucleotide sequences encoding one or more molecules may be used as well.

[0103] When the single polynucleotide construct containing more than one polynucleotide encoding a polypeptide is DNA, preferably, each polynucleotide encoding a polypeptide will be operably linked to a separate promoter. Alternatively, the polynucleotides encoding polypeptides may be operably linked to the same promoter in order to form a polycistronic transcription unit wherein each sequence encoding a polypeptide is separated by translational stop and start signals. Transcription termination is also shared by these sequences. While both DNA coding sequences are controlled by the same transcriptional promoter, so that a fused message (mRNA) is formed, they are separated by a translational stop signal for the first and start signal for the second, so that two independent polypeptides result. Methods of making such constructs are disclosed in U.S. Patent Nos. 4,713,339, and 4,965,196, which are herein incorporated by reference.

[0104] When the single polynucleotide construct containing more than one polynucleotide encoding a polypeptide is RNA, preferably, there will be

separate translational start and stop signals for each polypeptide-encoding sequence in order to produce two or more separate polypeptides.

[0105] In the present invention, the polynucleotide construct is complexed with one or more saponin derivatives of the invention by ionic interaction. Generally, the complex then contacts the cell membrane and is transfected into the cell, in a fashion analogous to "lipofection," a highly efficient transfection procedure, in which DNA or RNA is complexed with one or more cationic lipids for transfection into a cell. See Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417, (Nov. 1987); and Felgner *et al., Nature 337*:387-388 (1989).

[0106] In a formulation for preparing the saponin derivative/polynucleotide complexes of the invention, the saponin derivatives can be present at a concentration of between about 0.1 mole % and about 100 mole %, preferably about 5 mole % and 100 mole %, and most preferably between about 20 mole % and 100 mole %, relative to other compounds present in the formulation.

[0107] In preparing the saponin derivative/polynucleotide complexes of the invention, the polynucleotide construct can be solubilized in a buffer prior to mixing with one or more saponin derivatives. Suitable buffers include, for example, phosphate buffered saline (PBS), normal saline, Tris buffer, and sodium phosphate vehicle (100-150 mM preferred). Insoluble polynucleotides can be solubilized in a weak acid or base, and then diluted to the desired volume with a neutral buffer such as PBS. The pH of the buffer is suitably adjusted, and moreover, a pharmaceutically acceptable additive can be used in the buffer to provide an appropriate osmolarity.

[0108] The cationic saponin derivatives of the invention are present in solution as either monomers or as micelles, depending on the concentration of the saponin derivative, and on the ionic strength and pH of the solution. Because of their cationic nature, these derivatives tend to have critical micellar concentration values higher than those of the non-ionic derivatives such as alkylamide saponin derivatives. Cationic saponin derivatives can be prepared in water, isotonic solutions of 5% mannitol or sorbitol, or low ionic strength buffers, and mixed with the polynucleotide dissolved in a buffer solution

containing 0.15 M NaCl, mannitol or sorbitol, to form a saponin derivative/polynucleotide complex. Cationic saponin derivatives can also be used in conjunction with alkylamide saponin derivatives by mixing them together prior to adding the polynucleotide. Alternatively, the alkylamide saponin derivatives can be added to the cationic saponin derivative/polynucleotide complex to form a mixed micelles system containing the polynucleotide.

[0109] Cationic saponin derivatives of the invention with lipophilic chains containing 18 or more carbon atoms may form vesicles that are heterogeneous in size, particularly if they are mixed with alkylamide saponin derivatives having lipid chains containing 18 or more carbon atoms. Therefore, according to a preferred method, such cationic saponin derivatives are prepared by dissolution in a chloroform-methanol solvent mixture, and the resulting cationic saponin derivative/chloroform-methanol mixture is evaporated to dryness as a film on the inner surface of a glass vessel. On suspension in an aqueous solvent, these cationic saponin derivatives assemble themselves into vesicles. These vesicles are reduced to a selected mean diameter by means of a freeze-thaw procedure. Vesicles of uniform size can be formed prior to drug delivery according to methods for vesicle production known to those in the art; for example, the sonication of a lipid solution as described by Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987) and U.S. Pat. No. 5,264,618, which are herein incorporated by reference. Once the vesicles have been formed by suspension in aqueous solvent, they are added with stirring to the polynucleotide solution, to entrap the polynucleotide within the vesicles or to form a complex of cationic saponin and polynucleotide.

[0110] The saponin derivative/polynucleotide complexes of the invention may be delivered to any tissue, including, but not limited to, muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, or connective tissue. Preferably, the construct is delivered to muscle. The muscle may be skeletal or cardiac. Most preferably, the construct is delivered to skeletal muscle.

[0111] Preferably, the saponin derivative/polynucleotide complex is delivered to the interstitial space of tissues. "Interstitial space" comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels.

[0112] The saponin derivative/polynucleotide complexes can be administered by any suitable route of administration, including intramuscularly, subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (*i.e.*, across a mucous membrane, for example by direct application to mucosal surfaces either as drops or as aerosols). Similarly, the pharmaceutical composition of the present invention can by administered by any suitable route, including intramuscularly, into a cavity (e.g., intraperitoneally), subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (*i.e.*, across a mucous membrane, for example by direct application to mucosal surfaces either as drops or as aerosols).

[0113] Any mode of administration can be used. This includes needle injection, catheter infusion, biolistic injectors, particle accelerators (i.e., "gene guns", pneumatic "needleless" injectors, *e.g.*, Med-E-Jet (Vahlsing, H. *et al.*, *J. Immunol. Methods* 171:11-22 (1994)), Pigjet (Schrijver, R. *et al.*, *Vaccine* 15: 1908-1916 (1997)), Biojector (Davis, H. *et al.*, *Vaccine* 12:1503-1509 (1994); Gramzinski, R. *et al.*, *Mol. Med.* 4: 109-118 (1998))), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. The preferred mode is injection.

[0114] Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the animal, the precise condition requiring treatment and its severity, and the route

of administration. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

[0115] In humans, between 0.5 mg to 40 mg saponin derivative/polynucleotide complex is delivered. Preferably, between 1 mg and 10 mg saponin derivative/polynucleotide complex is delivered, with the polynucleotide comprising 10-15% w/w of the complex.

[0116] In certain embodiments, the saponin derivative/polynucleotide complexes are administered as a pharmaceutical composition. The pharmaceutical composition can be formulated according to known methods for preparing pharmaceutical compositions, whereby the substance to be delivered is combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their preparation are described, for example, in *Remington's Pharmaceutical Sciences*, 16th Edition, A. Osol, Ed., Mack Publishing Co., Easton, PA (1980), and Remington's Pharmaceutical Sciences, 19th Edition, A.R. Gennaro, Ed., Mack Publishing Co., Easton, PA (1995).

[0117] The pharmaceutical composition can be in the form of an emulsion, gel, solution, suspension, or other form known in the art. In addition, the pharmaceutical composition can also contain pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives. Administration of pharmaceutically acceptable salts of the saponin derivative/polynucleotide complexes described herein is preferred. Such salts can be prepared from pharmaceutically acceptable non-toxic bases including organic bases and inorganic bases. Salts derived from inorganic bases include sodium, potassium, lithium, ammonium, calcium, magnesium, and the like. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, basic amino acids, and the like.

[0118] For aqueous pharmaceutical compositions used *in vivo*, sterile pyrogen-free water is preferred. Such formulations will contain an effective amount of the substance together with a suitable amount of vehicle in order to

prepare pharmaceutically acceptable compositions suitable for administration to a human or animal.

[0119] A pharmaceutical composition can be in solution form, or alternatively, in lyophilized form for reconstitution with a suitable vehicle, such as sterile, pyrogen-free water. Both liquid and lyophilized forms will comprise one or more agents, preferably buffers, in amounts necessary to suitably adjust the pH of the injected solution.

[0120] The container in which the pharmaceutical formulation is packaged prior to use can comprise a hermetically sealed container enclosing an amount of the lyophilized formulation or a solution containing the formulation suitable for a pharmaceutically effective dose thereof, or multiples of an effective dose. The pharmaceutical formulation is packaged in a sterile container, and the hermetically sealed container is designed to preserve sterility of the pharmaceutical formulation until use. Optionally, the container can be associated with administration means and or instruction for use.

[0121] In certain embodiments of the invention, the saponin derivative/polynucleotide complexes are delivered with additional antiviral agents. Antiviral agents include, but are not limited to, protease inhibitors, nucleoside RT inhibitors, non-nucleoside RT inhibitors, fusion/binding inhibitors, and pyrophosphate analogue RT inhibitors.

[0122] Typical vaccines using the saponin derivatives of the invention include viral vaccines, such as influenza, herpes, cytomegalovirus, HIV-1, HTLV-1, FIV, cancer vaccines, and parasitic vaccines.

[0123] Applications of the present invention include vaccination against viruses in which antibodies are known to be required or to enhanced viral infection. There are two strategies that can be applied here. One can specifically target the cellular pathway during immunization thus eliminating the enhancing antibodies. Alternatively one can vaccinate with the gene for a truncated antigen which eliminate the humoral epitomes which enhance infectivity. The use of DNA or mRNA vaccine therapy could similarly provide a means to provoke an effective cytotoxic T-cell response to weakly antigenic tumors.

[0124] A second application is that this approach provides a method to treat latent viral infections. Several viruses (for example, Hepatitis B, HIV and members of the Herpes virus group) can establish latent infections in which the virus is maintained intracellularly in an inactive or partially active form. There are few ways of treating such an infections. However, by inducing a cytolytic immunity against a latent viral protein, the latently infected cells will be targeted and eliminated.

[0125] A related application of this approach is to the treatment of chronic pathogen infections. There are numerous examples of pathogens which replicate slowly and spread directly from cell to cell. These infections are chronic, in some cases lasting years or decades. Examples of these are the slow viruses (e.g. Visna), the Scrapie agent and HIV. One can eliminate the infected cells by inducing an cellular response to proteins of the pathogen.

[0126] Finally, this approach may also be applicable to the treatment of malignant disease. Vaccination to mount a cellular immune response to a protein specific to the malignant state, be it an activated oncogene, a fetal antigen or an activation marker, will result in the elimination of these cells.

[0127] The use of saponin derivatives of the invention with DNA/mRNA vaccines could in this way greatly enhance the immunogenicity of certain viral proteins, and cancer-specific antigens, that normally elicit a poor immune response. The mRNA vaccine technique should be applicable to the induction of cytotoxic T cell immunity against poorly immunogenic viral proteins from the Herpes viruses, non-A, non-B hepatitis, and HIV, and it would avoid the hazards and difficulties associated with in vitro propagation of these viruses. For cell surface antigens, such as viral coat proteins (e.g., HIV gp120), the antigen would be expressed on the surface of the target cell in the context of the major histocompatibility complex (MHC), which would be expected to result in a more appropriate, vigorous and realistic immune response.

[0128] Finally, in the case of the DNA/mRNA vaccines, the protein antigen is never exposed directly to serum antibody, but is always produced by the transfected cells themselves following translation of the mRNA. Hence, anaphylaxis should not be a problem. Thus, the present invention permits the

patient to be immunized repeatedly without the fear of allergic reactions. The use of the DNA/mRNA vaccines with the saponin derivatives of the present invention makes such immunization possible.

[0129] Parenteral or transmucosal administration to an animal of coding or noncoding bacterial DNA complexed with cationic saponin derivatives stimulates a non-specific innate immunity with the production of cytokines and natural killer (NK) cells with anti-tumor activity and effective in the treatment of cancer. Formulations of bacterial DNA:cationic saponin derivatives in combination with an antigen would stimulate a specific humoral and T-cell immune response against such antigen and useful in the development of preventive and therapeutic vaccines. Bacterial DNA:cationic saponin derivatives can also be administered in combination with other immune modulatory compounds such as QS-21, GPI-0100, immune stimulatory polysaccharides and their derivatives, monophosphoryl lipid A (MPL), muramyl dipeptides (MDP), alum, and others, to provide a synergistic response.

[0130] Having now generally described the invention, the same will become more readily understood by reference to the following specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

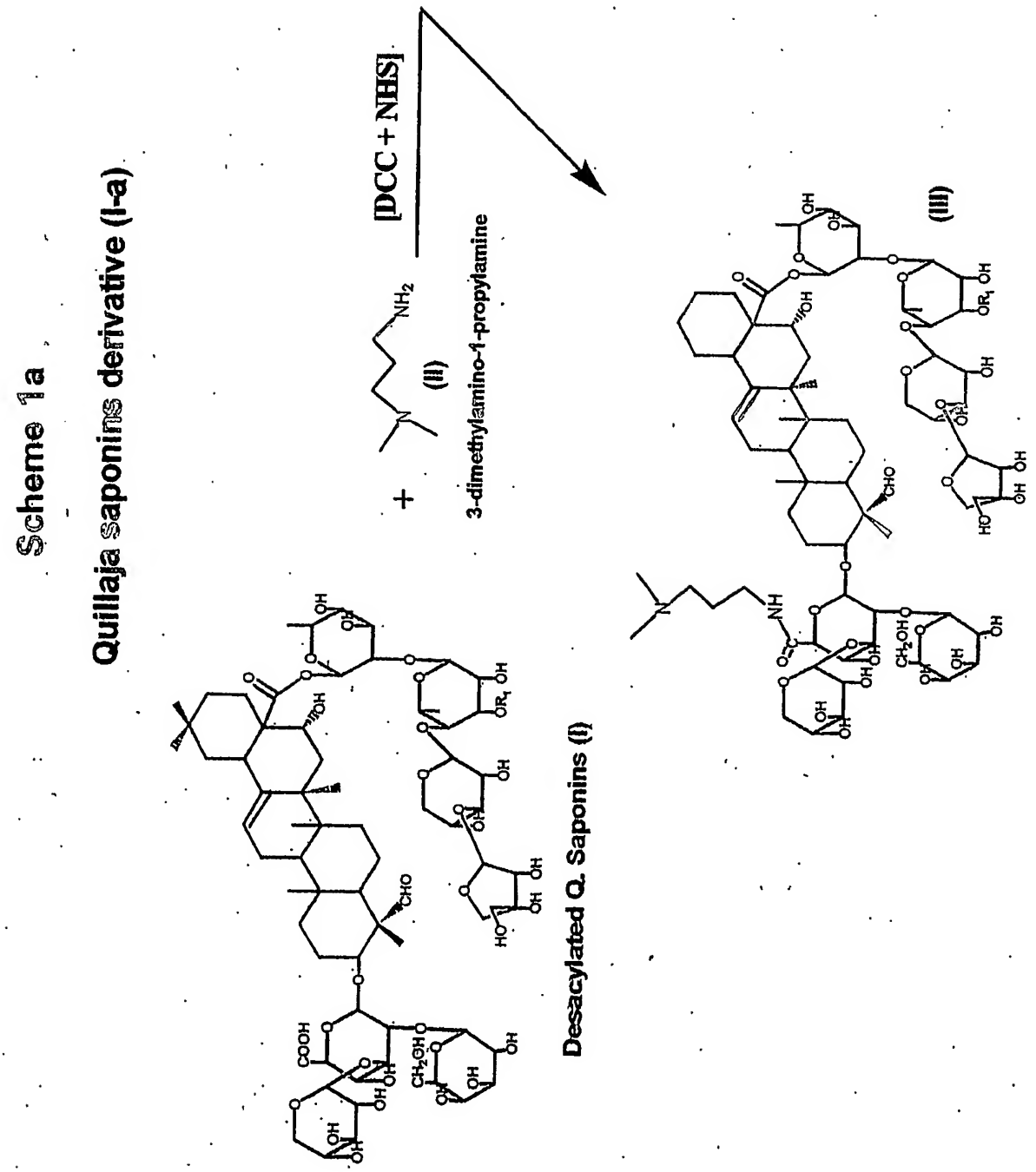
EXAMPLES

Example 1

Preparation of Group 1 Saponin Derivatives

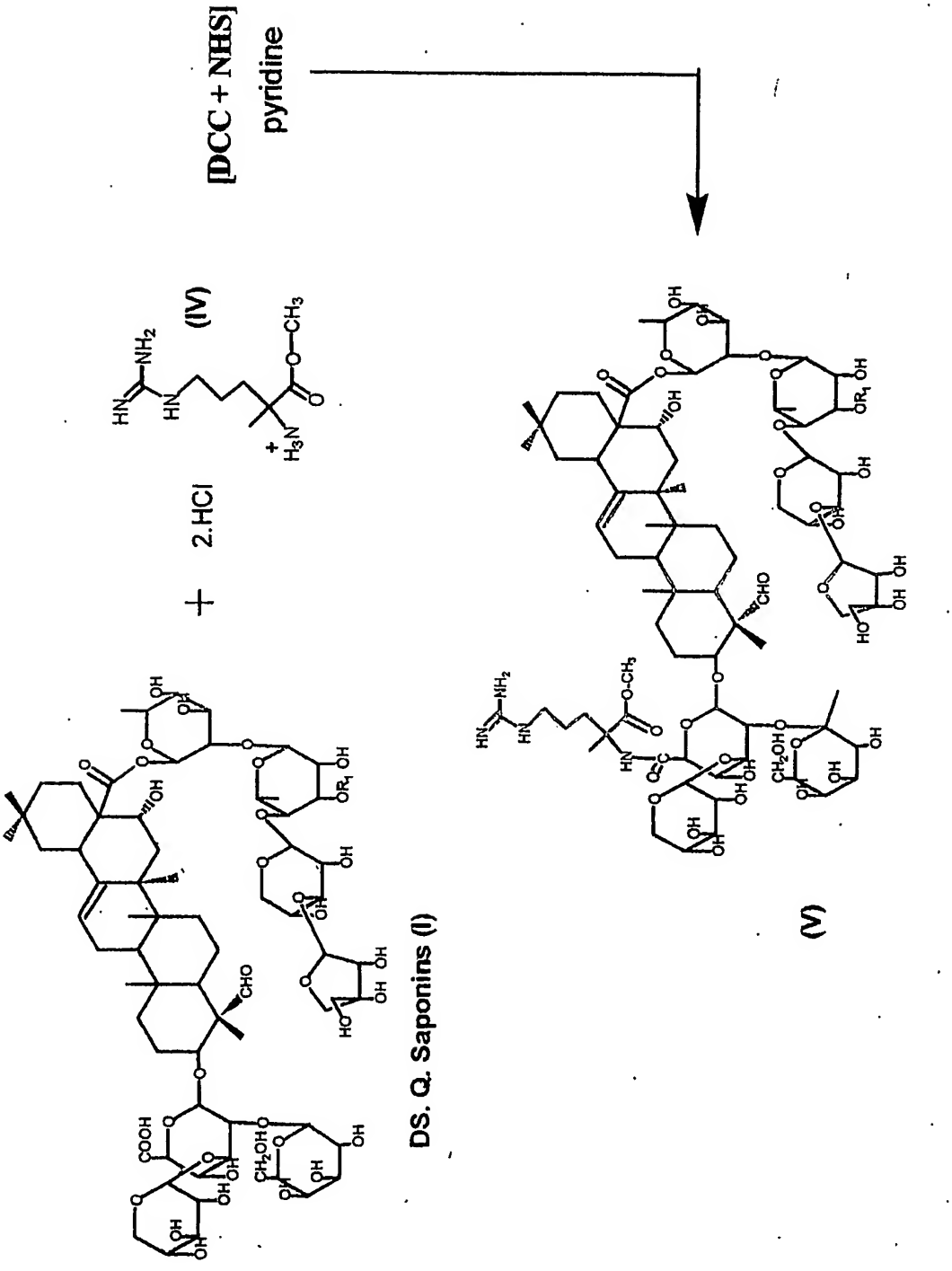
[0131] Schemes 1a-1c illustrate the syntheses of compounds a-c, respectively, as described below.

a) 3-dimethylamino-l-propylamine-saponin derivative. Hydrolyzed or deacylated (DS) quillaja saponin (compound I in Scheme 1a), gypsophylla saponin or a similar one (2.5 g, ~ 1.5 mmol) was dissolved in dry


Scheme 1a
Quillaja saponins derivative (I-a)
[DCC + NHS]
(II)
3-dimethylamino-1-propylamine
Desacylated Q. Saponins (I)
(III)

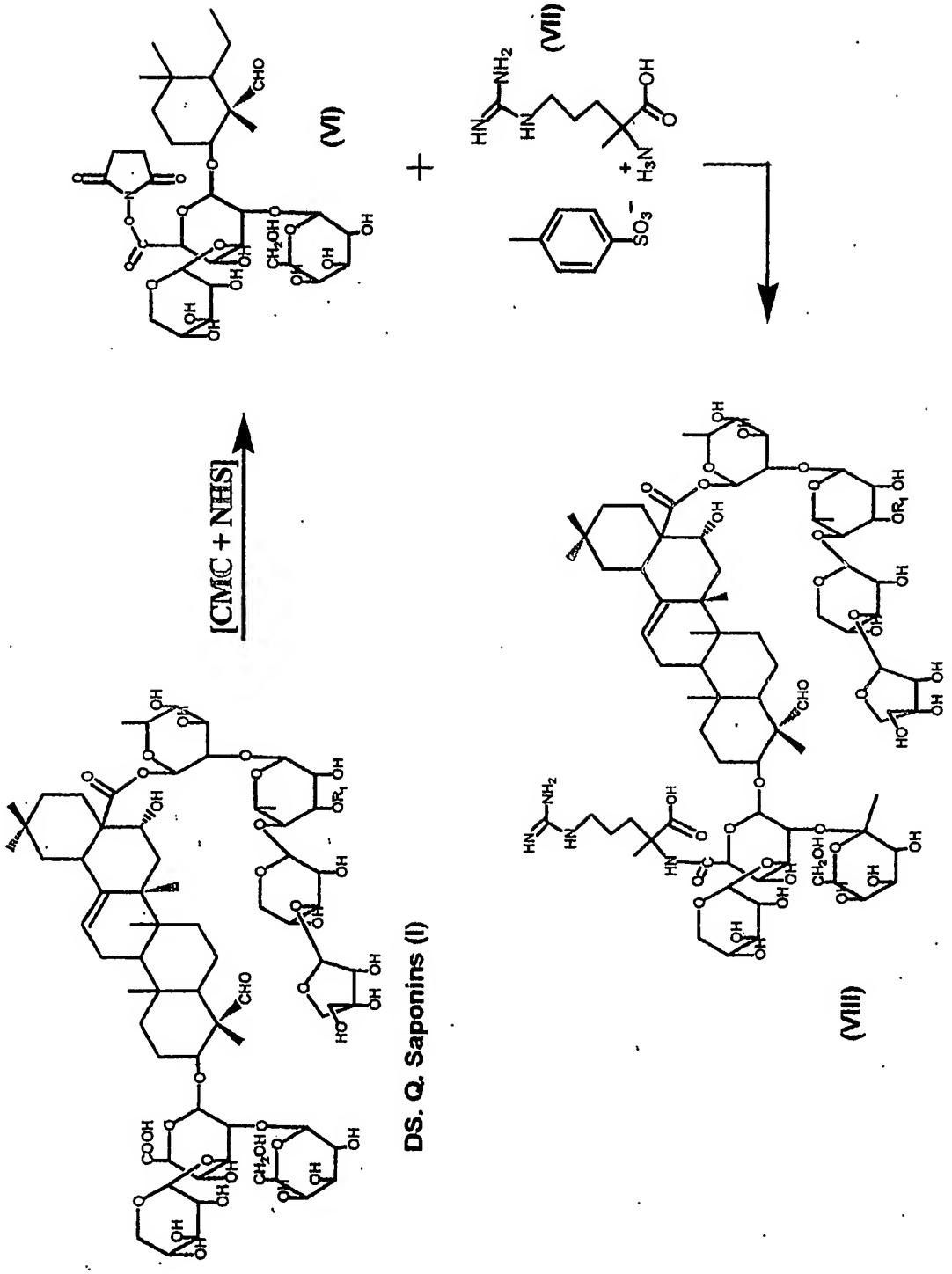
Scheme 1b

Quillaja saponin-arginine methyl ester derivative (1-b)



Scheme 1c

Quillaja saponin-arginine derivative (1-c)



pyridine (25 mL), and 1,3-dicyclohexyl carbodiimide (DCC) (0.93 g, 4.5 mmol), and N-hydroxy succinimide (NHS) (0.52 g, 4.5 mmol), each dissolved in 12.5 mL of pyridine, were added with vigorous stirring. Subsequently, 3-dimethylamino-1-propylamine (0.46 g, 4.5 mmol) (II) dissolved in pyridine (10 mL) was added dropwise and with stirring over a period of 30 min. The reaction was allowed to proceed with stirring for 2 days. Glacial acetic acid (0.2 mL) was added to the reaction, and the mixture was stirred overnight at room temperature. The resulting suspension was added to distilled water (200 mL) and stirred overnight. Precipitated material (mostly *N,N'* -dicyclohexyl urea) was collected by filtration. The filtrate was evaporated on a rotary evaporator to remove the pyridine.

[0132] The resulting syrup containing the derivatized saponin was diluted with water, put in dialysis bags (M.W. cut off ~ 3,000), and dialyzed against several changes of an aqueous solution of 40 mmolar acetic acid for four days. The resultant precipitate was filtered and the clear solution was shelled and lyophilized to get the powdered saponin 3-dimethylamino-1-propylamide derivative (III). The preparation can be further purified by reverse phase chromatography on RP-18 or one similar.

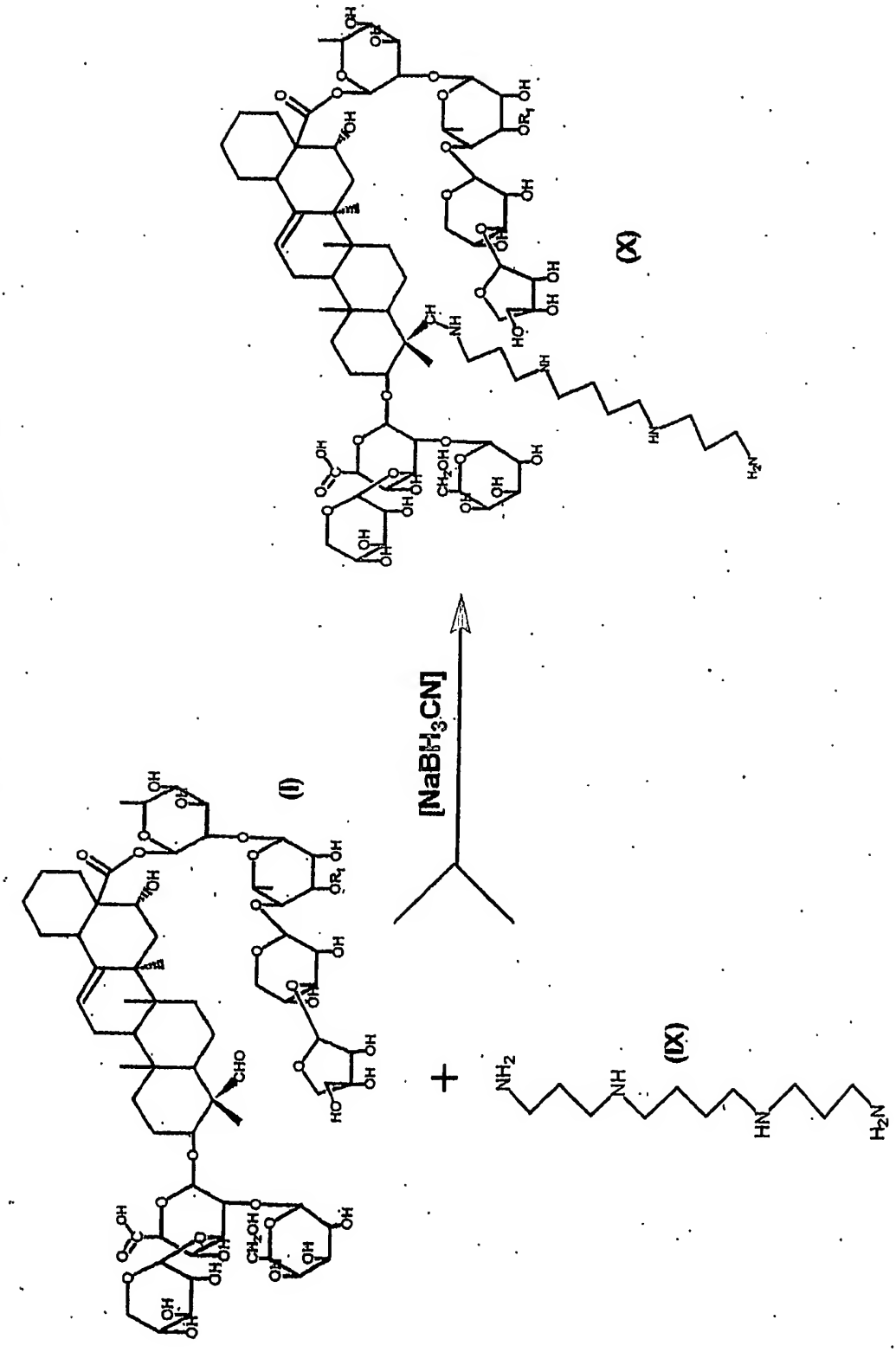
b) Arginine methyl ester-saponin derivative. To 2.5 gm (~ 1.5 mmoles) of DS quillaja saponins (I) dissolved in 25 mL of pyridine, was added 4.5 mmoles (0.93 g) of dicyclohexylcarbodiimide (DCC) and 4.5 mmoles (0.52 g) of *N*-hydroxysuccinimide (NHS), each dissolved in 12.5 mL of pyridine each. To the reaction mixture 0.8 g of L-arginine methyl ester dihydrochloride (IV) (~3.0 mmol) dissolved in methanol (10 mL) was added drop wise with stirring over a period of 2 hours. The reaction was then allowed to proceed with stirring for 2 days. Glacial acetic acid (0.2 mL) was added to the reaction, and the mixture was stirred overnight at room temperature. The resulting suspension was added to distilled water (250 mL) and stirred overnight. Precipitated material (mostly *N,N'* -dicyclohexyl urea) was removed by filtration. The clear filtrate was evaporated on a rotary evaporator to remove the pyridine. The resulting syrup containing the derivatized saponin was diluted with water put in dialysis bags (M.W. cutoff ~

3,000) and dialyzed against several changes of 40 mmolar acetic acid for 3 days. Continue dialysis against several changes of water, filter the dialyzed solution, and lyophilize to obtain the dry arginine methyl ester-saponin (V).

c) Arginine-saponin derivative using water-soluble carbodiimide. Two (2) g of purified desacylsaponins (I) (~1.20 mmoles) were dissolved in 20 ml of pyridine at room temperature, and added with mixing about 1.50 mmoles of CMC (0.64 g of CMC and 2.0 mmoles of N-hydroxysuccimide (0.23 g of NHS). If needed, more pyridine may be added to dissolve the reactants. The reaction was allowed to proceed with mixing overnight at room temperature under anhydrous conditions. Most of the pyridine was removed by rotary evaporation at room temperature. Added to the syrupy residue was 250 ml, of isopropanol to precipitate the saponin intermediate (VI) and collect it by filtration. The ppt. was washed on filter paper with isopropanol to remove the excess of CMC, CMC urea and NHS. The intermediate (VI) (~ 1.20 mmoles) was dissolved in ~ 25 mL of 50% pyridine and added to 0.45 g (~ 2.5 mmole) of L-arginine (2-amino-5-guanidinopentanoic acid) (VII) dissolved in 15 ml of water plus *p*-toluenesulfonic acid adjusted to pH ~ 7. Reacted with mixing for 24 hours at room temperature yielded the saponin analog with an arginine side chain (VIII). If needed the pH of the reaction can be adjusted to ~ 7-8 by the addition of aqueous 4 M *p*-toluenesulfonic acid. In a rotary evaporator the pyridine was removed from the reaction mixture, the syrupy residue was dissolved in 40 mM acetic acid and dialyzed against several changes of this solution for 2 days to remove free arginine. Dialyze against several changes of water, filter, and lyophilize to obtain the dry arginine-saponin derivative (VIII).

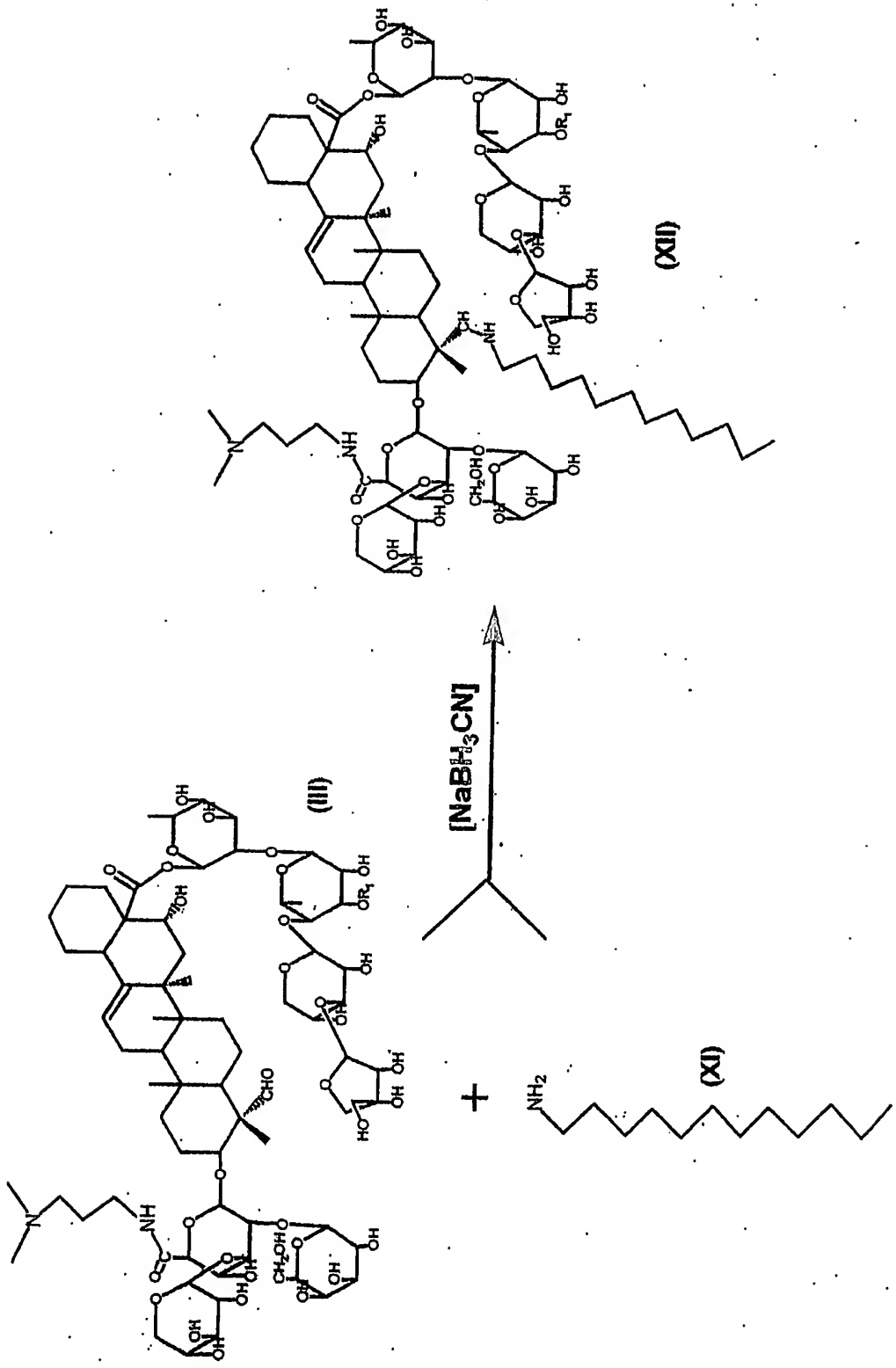
Scheme 2

Quillaja saponins derivative (2-a)



Scheme 3

Quillaja saponins derivative (3-a)



Example 2

Preparation of Group 2 Saponin Derivatives

[0133] Scheme 2 illustrates the synthesis of compound a, as described below.

a) Saponin-spermine aldehydic derivative. To 1.2 g of spermidine (IX) (6 mmoles) dissolved in 50 mL of aldehyde-free methanol, adjusted to pH ~ 9 with acetic acid, and containing 0.12 g of Na cyanoborohydride (~ 2 mmoles) over a 4 hour period add dropwise with stirring 2 g of desacylsaponins (I) (~ 1.20 mmoles) dissolved in 20 ml of 50% pyridine. The reaction was allowed to proceed for 72 hours to allow the formation of an imine between the spermine primary amines and the triterpenoid aldehyde and its subsequent reduction by Na cyanoborohydride to form a stable secondary amine linkage (X). The reaction mixture was dialyzed against water, followed by dialysis against several changes of 10 mM acetic acid, and lyophilized.

Example 3

Preparation of Group 3 Saponin Derivatives

[0134] Scheme 3 illustrates the synthesis of compound a, as described below.

a) 3-dimethylamino-1-propylamine-dodecylamine saponin derivative. The 3-dimethylamino-1-propylamine-saponin derivative (III) was prepared as described under 1-a. To 1.1 g of dodecylamine (XI) (6 mmole) dissolved in 50 mL of 50% dimethylformamide, pH ~ 8-9, and containing 0.12 g of Na cyanoborohydride (~ 2 mmoles), 2 g of derivative (III) (~1.20 mmoles) dissolved in 20 ml of 50% pyridine were added dropwise with stirring over a 4 hour period. The reaction was allowed to proceed for 48 hours to allow the formation of an imine between a dodecylamine and the triterpenoid aldehyde and its subsequent reduction by Na cyanoborohydride to form a stable secondary amine linkage. The reaction mixture was poured into 1 L of isopropanol to precipitate the 3-dimethylamino-1-propylamine-saponin-

dodecylamine derivative (XII). The precipitated material was collected by filtration, washed with isopropanol, dissolved in a minimal volume of 0.1 M acetic acid and dialyzed against several changes of 40 mM acetic acid, followed by dialysis against 10 mM acetic acid. Precipitated material was removed and the clear solution lyophilized.

Example 4

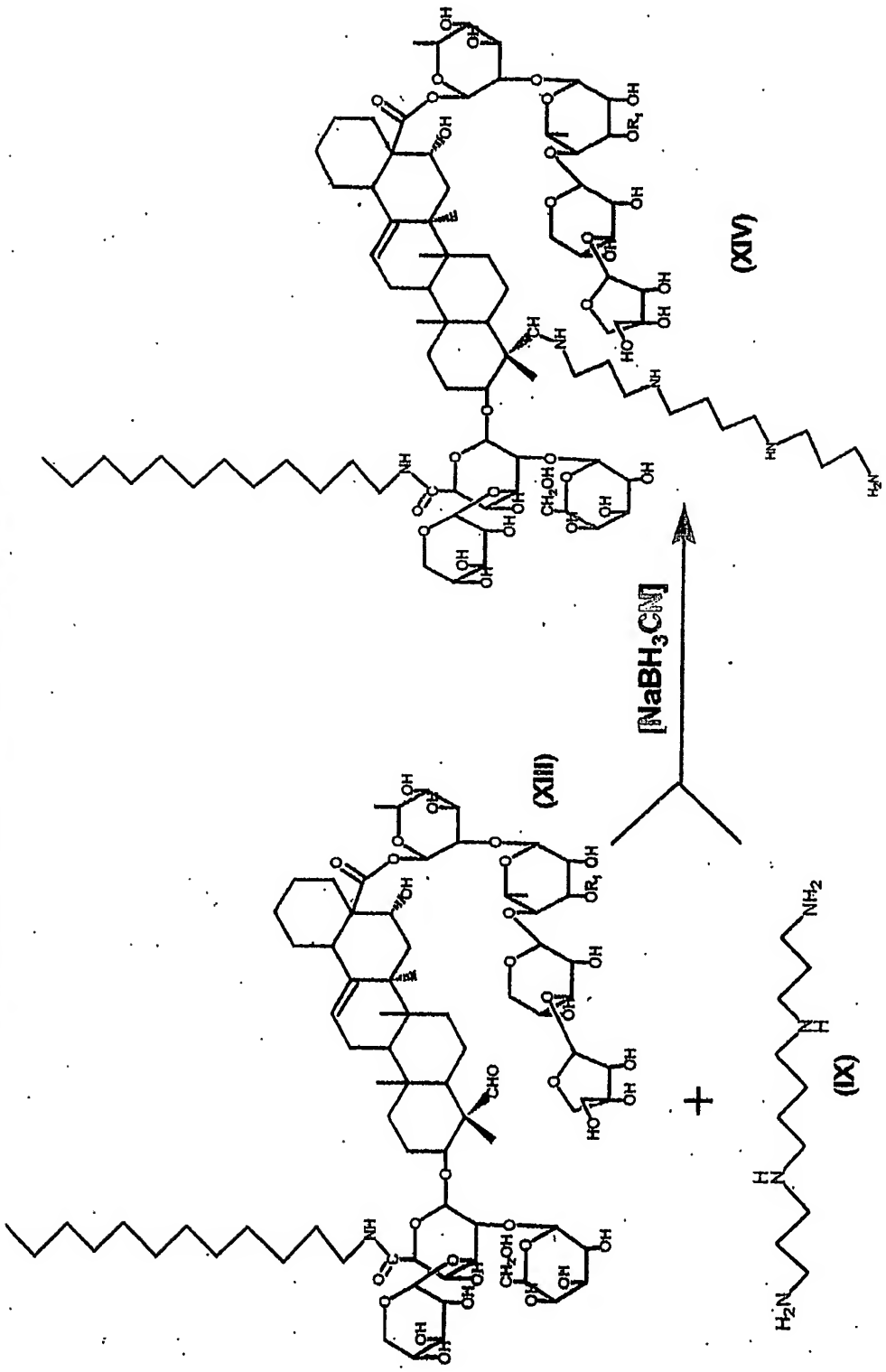
Preparation of Group 4 Saponin Derivatives

[0135] Scheme 4 illustrates the synthesis of compound a, as described below.

a) Dodecyl amide saponin-spermine aldehydic derivative. To D.S. quillaja saponin, gypsophylla saponin or a similar one (2.5 g, ~ 1.5 mmol) dissolved in dry pyridine (25 mL), were added with vigorous stirring 1,3-dicyclohexyl carbodiimide (DCC) (0.93 g, 4.5 mmol), and *N*-hydroxy succinimide (NHS) (0.52 g, 4.5 mmol), each dissolved in 12.5 mL of pyridine. Subsequently, dodecylamine (0.83 g, 4.5 mmol) dissolved in pyridine (10 mL) was added dropwise and with stirring over a period of 30 min. The reaction was allowed to proceed with stirring for 2 days. Glacial acetic acid (0.2 mL) was added to the reaction, and the mixture was stirred overnight at room temperature. The resulting suspension was added to distilled water (200 mL) and stirred overnight. The precipitated material (mostly *N,N'* -dicyclohexyl urea) was removed by filtration. The clear filtrate was evaporated on a rotary evaporator to remove the pyridine. The resulting syrup containing the derivatized saponin was diluted with water delivered into dialysis bags (M.W. cut off ~12,000) and dialyzed against several changes of an aqueous solution of 40 mmolar acetic acid for four days. The resulting precipitate was filtered and the clear solution was shelled and lyophilized to get the dry dodecylamide saponin derivative (XIII). To 1.2 g of spermidine (IX) (6 mmoles) dissolved in 50 mL of aldehyde-free methanol, and containing 0.12 g of Na cyanoborohydride (~2 mmoles) add dropwise with stirring and over a 6-8 hours period 2 g of dodecylamide saponin (XIII) (~1.20 mmoles) dissolved in

Scheme 4

Quillaja saponins derivative (4-a)



20 ml of aldehyde-free methanol. The reaction was allowed to proceed for 72 hours to allow the formation of an imine between the spermine primary amines and the triterpenoid aldehyde and its subsequent reduction by Na cyanoborohydride to form a stable secondary amine linkage. The dodecyl amide saponin-spermine aldehydic derivative (XIV) was separated from the excess reactants by gel filtration on Sephadex G-15, using water as an eluent. The void volume peak containing the derivative (XIV) was lyophilized. Further purification can be achieved by reverse chromatography on Silica RP-18 using a methanol-water gradient with 50 mM acetic acid, or using ion exchange chromatography using a DEAE matrix and a salt gradient at acid pH. Collect fraction containing the derivative (XIV) and remove the salt by gel filtration on Sephadex G-15 using water as eluent and lyophilize the void volume peak containing (XIV).

Example 5

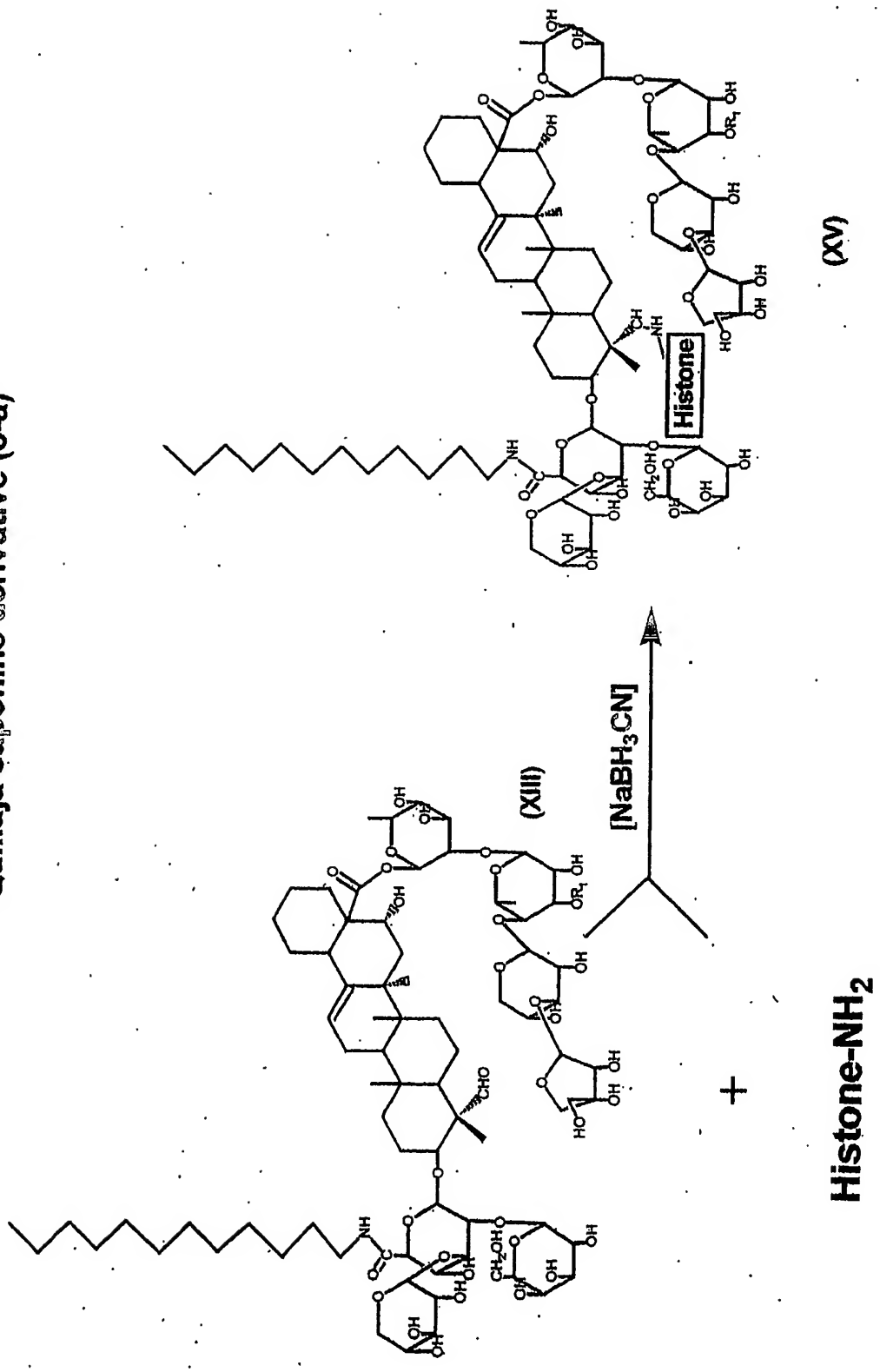
Preparation of Group 5 Saponin Derivatives

[0136] Schemes 5a-5c illustrate the syntheses of compounds a-c, respectively, as described below.

a) Histone-dodecylamide saponin derivatives. To 1.10 g (~ 10 mmoles a.a., ~ 1-2 mmoles NH_2)) of histones dissolved in 40 mL of 6 M urea, 0.1 M HEPBS buffer pH 8.0, were added 0.5 mmole (0.9 g) of the dodecylamide saponin derivative (XIII) dissolved in 10 mL of pyridine, and 0.3 g (0.5 mmole) of Na cyanoborohydride dissolved in 5-10 ml pyridine, and allowed to react with stirring for 72 hours at room temperature. The reaction mixture was dialyzed against several changes of water to remove the excess of reactants. To the dialyzed solution, containing some precipitated material, acetic acid was added to re-dissolve the historic derivative (XV). After filtration, the clear solution was lyophilized to recover the derivative (XV). The histone derivative had a degree of substitution ~ 0.05 or 1 residue of dodecylamide saponin derivative for every 20 amino acid residues.

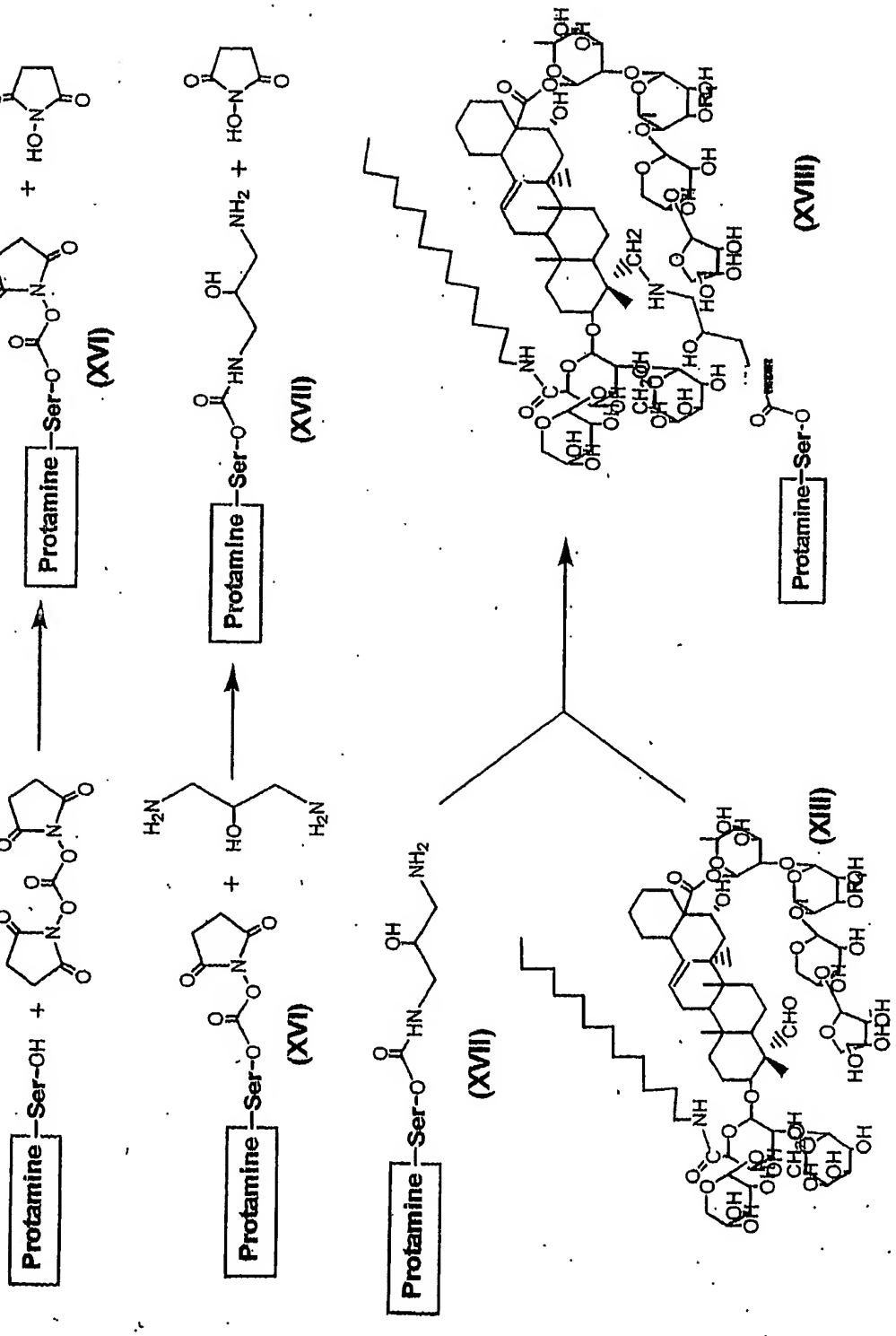
Scheme 5a

Quillaja saponins derivative (5-a)

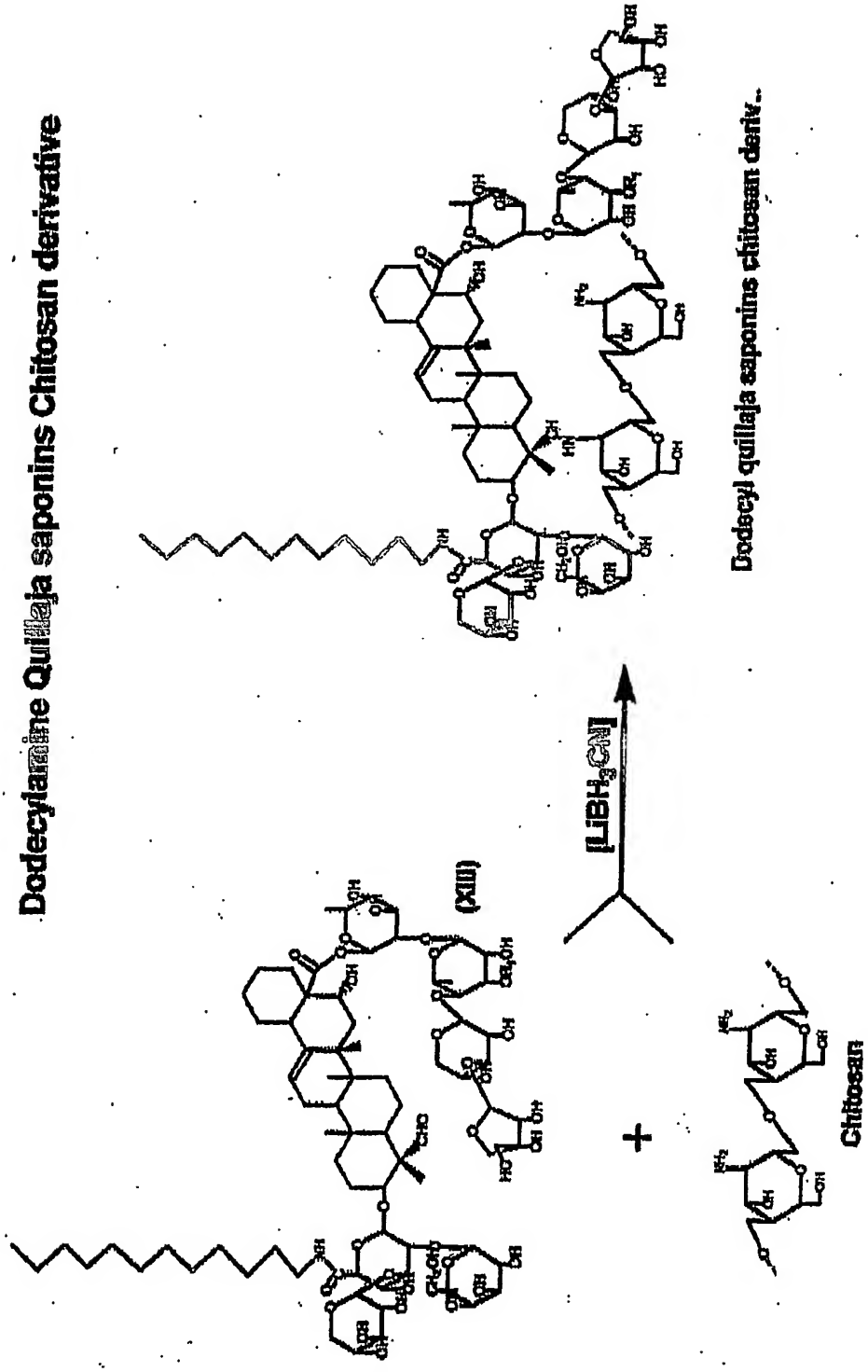


Scheme 5b

Quillaja saponins derivative (5-b)



Scheme 5c


Dodecylamine Quillaja saponins Chitosan derivative
(XIII)
[LiBH3CN]
Dodecyl quillaja saponins chitosan deriv..
Chitosan

Alternatively, the histone dodecylamide derivative (XV) was separated from the excess reactants by gel filtration of Sephadex G-25 (medium) equilibrated with 20 mM acetic acid. To the reaction enough acetic acid was added in a chemical hood to adjust the concentration to 20 mM acetic acid and stirred for ~ 1 hour. The reaction mixture was applied to the Sephadex G-25 column and eluted with 20 mM acetic acid. The void volume was collected and lyophilized to recover the histone derivative (XV). The histone derivative had a degree of substitution (d.s.) ~0.05 or 1 residue of dodecylamide saponin derivative for every 20 amino acid residues. The d.s. was determined by estimation of the histones and saponin concentration using the biuret and anthrone reactions for protein and carbohydrate respectively.

b) Protamine-dodecylamide Q. saponin derivatives. To 1 g of protamine, (salmine-free base containing~ 9 mmoles a.a., ~ 0.74 mmoles serine), dissolved in 25 mL of anhydrous dimethylsulfoxide, were added with stirring 0.12 g (0.45 mmoles) of *N,N'*-disuccinimidyl carbonate dissolved in 2 ml of DMSO. To this mixture was added slowly and with stirring 0.055 g (0.5 mmoles) of 4-dimethylaminopyridine dissolved in 1 mL of dioxane or dimethylformamide and reacted overnight at room temperature to form the succinimidyl carbonate-protamine intermediate (XVI) with a d.s. ~ 0.05. To the intermediate (XVI), 0.36 g of 1,3-diamino-2-propanol (a 8x excess over the succinimyl carbonate) dissolved in 2 mL DMSO were added, and the reaction was allowed to continue with stirring for 48 at RT hours to form a protamine-propylamine derivative (XVII). The intermediate (XVII) was precipitated over night by adding the reaction with stirring into 400-500 mL of acetone with 5% glycerol. The precipitate was collected by filtration, and washed by gravity or gentle suction with several volumes of acetone glycerol (95v/5v). The collected material was not allowed to get dry. To the precipitated intermediate (XVII) dissolved in 40 mL of freshly prepared 8 M urea solution, 0.1 M HEPBS buffer pH 8.0, were added 1 mmole (1.8 g) of dodecylamide saponin derivative (XIII) dissolved in 10 mL of pyridine, and 0.4 g (0.67 mmole) of Na cyanoborohydride dissolved in 5-10 ml pyridine. The reaction was allowed to continue with stirring for 72 hours at R.T. The

reaction mixture was dialyzed against several changes of water, followed by 20 mM acetic acid to remove excess of reactants, filtered and lyophilized to recover the histone derivative (XVIII). The (XVIII) derivative had a d.s. ~0.05 or 1 residue of dodecylamide saponin derivative for every 20 amino acid residues. Alternatively, the excess of reactants was separated from the protamine-propylamine derivative (XVII) by gel filtration on Sephadex G-25. To the reaction mixture in DMSO enough urea was added to make it ~6M and apply it to a Sephadex G-25 column equilibrated with 40 mM acetic acid and separate (XVII) from the excess reactants by eluting with 40 mM acetic acid. The void volume containing the protamine derivative was collected, concentrated on a rotary evaporator and lyophilized. The lyophilized material was dissolved in 40 mL of freshly prepared 8 M urea solution, 0.1 M HEPBS buffer pH 8.0, and 1 mmole (1.8 g) of dodecylamide saponin derivative (XIII) dissolved in 10 mL of pyridine, and 0.4 g (0.67 mmole) of Na cyanoborohydride dissolved in 5-10 ml pyridine were added. The reaction was allowed to continue with stirring for 72 hours at R.T. The reaction mixture was dialyzed against several changes of water. Any formed precipitate was re-dissolved by adding to the protamine solution acetic acid to adjust the pH to - 7. The solution was filtered and lyophilized to recover the histone derivative (XVIII). The (XVIII) derivative had a degree of substitution ~ 0.05 or 1 residue of dodecylamide saponin derivative for every 20 amino acid residues. The d.s. was determined by estimation of the histones and saponin concentrations using the biuret and anthrone reactions for proteins and carbohydrates respectively.

c) Dodecylamine-saponin-chitosan derivative. Commercial crab or shrimp chitosan (~85% deacetylated) is further deacetylated by autoclaving a suspension of chitosan (10% w/v) in 40% (10N) NaOH at 120°C for 3 hours. After 3 hours the NaOH containing reaction is dissolved with water 10 fold and the chitosan is left to sediment overnight. Discard the supernatant and wash the deacetylated chitosan several times by decantation with 10-20 volumes of water to bring the pH ~9. Re-suspend the chitosan in ethanol, collect and wash on filter paper, and store over desiccant.

[0137] The chitosan molecular weight ~200,000 to 300,000, is fragmented using hydrogen peroxide (Chang KL B, *et al., J. Agric Food Chem 49*:4845-51 (2001)). To chitosan dissolved in 2% acetic acid to yield a 1% solution, hydrogen peroxide (30% w/w) is added to yield a final concentration of 1.5% (dilution 1:20) and the reaction mixture is left to react at 50°C for about 5 hours. After 5 hours, the reaction is cooled in an ice bath. The average molecular weight of the fragmented chitosan should be around 12,000. Add to the reaction Chelex 100 resin, about 0.2 g per 100 mL reaction, to sequester the metal ions and stop the reaction. Remove the resin by filtration and dialyze the reaction mixture using a 12,000 M.W. cut off membrane against several changes of 0.2 M acetic acid to remove the hydrogen peroxide and small M.W. oligosaccharides. Lyophilize the dialyzed material.

[0138] To 1 g of fragmented chitosan (6.2 mmoles glcN) dissolved in 0.1 M acetic acid add 1 g (~0.6 mmoles) of GPI-0100, and bring the pH to ~5 with LiOH. Add to the reaction mixture 0.1 mmole of Li cyanoborohydride and let react for 72 hours with gentle stirring. The product is precipitated by addition of 10 volumes of ethanol or isopropanol. Wash the precipitated material with ethanol, dissolve it in a minimal volume of 0.1 M acetic acid, adjust the pH to 5 with LiOH, and re-precipitate with 10 vol. of ethanol. Collect and wash with ethanol over filter paper and store over dessicant. The product is analyzed by reverse phase HPLC using a acetonitrile-water gradient at pH~9. The degree of submission is to be determined colorimetrically from the differential between the amino groups before and after modification using the TNBS reaction.

d) Polyethylenimine quillaja saponin derivative. To 2 g of DS quillaja saponins (I) (1.2 mmoles) dissolved in 50 mL of anhydrous pyridine were added, with vigorous stirring, 0.744 g DCC (3.6 mmoles) and 0.412 g NHS (3.6 mmoles), and the mixture was stirred for 30 minutes. To this mixture, 1.42 mL of polyethylenimine (linear, MW of approximately 423) (3.6 mmoles) dissolved in 50 mL pyridine was added dropwise over a period of 30 minutes. The reaction was allowed to proceed for 72 hours at RT and then concentrated to about 25 mL in a rotary evaporator. To the resulting

suspension, 50 mL of distilled water was added, the suspension was stirred overnight, and the precipitated material removed by filtration. The clear filtrate was evaporated in a rotary evaporator to remove the pyridine. The syrupy residue was dissolved in 15 mL of distilled water, filtered and dialyzed for 3 days against water using a membrane with a molecular weight cut off of 3500. The dialyzed material was filtered to remove any insoluble matter, frozen in a dry ice/isopropanol bath and freeze-dried to recover 1.43 g of the polyethylenimine quillaja saponin derivative. The derivative was analyzed by HPLC using a Vydac C4 column.

Example 6

High Performance Liquid Chromatography (HPLC) Purification of Saponin Derivatives

[0139] Final preparations of small molecular weight derivatives, molecular weights up to 5000, (~ 100 to 20 μg) were analyzed by reverse phase HPLC using a Vydac C4 column (5 μm particle size, 300 Å pore size, 0.46 x 25 cm), eluted with a water/acetonitrile linear gradient between 10 to 40% acetonitrile and using a flow rate of 1 mL/min. Under certain conditions, the eluent contained 0.1% diethylamine to limit the ionization of the cationic groups of the derivatives. Effluent was monitored at 214 nm.

Example 7

Chromatographic Analysis of High Molecular Weight Glycoside/saponin-polymer Conjugates

[0140] Glycoside/saponin conjugates containing high molecular weight polymers, such as proteins, polylysine, and similar cationic polymers were analyzed by one of the following procedures:

i) Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, using a gel containing 8-10% acrylamide, 0.1% SDS and 0.1 M Na phosphate pH ~ 7.

ii) gel filtration using 6 M urea/0.5 M acetic acid or 50% dimethylsulfoxide as an eluent.

iii) ion-exchange chromatography on a carboxymethylated matrix using a NaCl salt gradient in 6 M urea at pH ~ 4.50

Example 8

Testing of Immune Stimulatory Effect on DNA Vaccines Using a DNA Plasmid for OVA.

[0141] The immune stimulatory effect of a compound over the immune response elicited by DNA vaccination can be assessed by the antibody response against a transiently expressed antigen encoded by a DNA or RNA sequence. An indication of the modulatory effects of a compound on the type of immune response can be obtained from the stimulation of the different antibody isotypes. In effect, production in mice of the IgG2a isotype has been associated with Th1 immunity, while a predominant IgG1 response is a good indicator of Th2 immunity.

[0142] The immune stimulatory effect of some compounds was determined by the increase of anti-OVA antibodies after immunization with a DNA plasmid for OVA in the presence and absence of such compounds. Female BALB/c mice of approximately 6 to 9 weeks of age were immunized intramuscularly on days 1 and 15 with 50 or 100 μg of the compounds being tested. Injections were given in two sites (50 μL/site) in a total volume of 100 μL. Mice injected with PBS only were used as negative controls. Sera was collected on days 29, 50 and 71 and assayed for anti-OVA antibodies by ELISA using Immunion II plates coated overnight at 4 °C with 100 μL per well of an OVA solution (50 μg/mL). Plates were washed twice with PBS and non-specific binding prevented by incubating all the wells for 1.5 hour at 37 °C with 100 μL of 2% casein hydrolysate in PBS. Plates were washed 4 times with 0.05% Tween 20 in distilled water. The initial sera dilution used was 1:30 and samples were diluted serially 1:2 thereafter. Sera dilutions were incubated for 1.5 hours at 37 °C, plates washed and incubated with anti-IgG-HPR

conjugates, washed and developed with a TMB substrate for 15 minutes at room temperature, and the reaction stopped by addition of 0.18 M sulfuric acid. Titers were determined at 450 nm using a cut-off value of 0.1 O.D.

[0143] FIGs. 3 and 4 illustrate the results of use of this protocol to measure the immune stimulatory effect of GPI-0330 and GPI-0332 on the IgG1 and IgG2a production in BALB/c mice.

Example 9

Immunization of Balb/c Mice with OVA cDNA in the Presence and Absence of 3-dimethylamino-1-propylamino-DS-saponin (DMPS)

[0144] Female Balb/c mice were immunized intramuscularly at days 1 and 15 with 0.2 mL of phosphate buffered saline solution (PBS) containing 20 μg of chicken OVA cDNA alone or with 50 μg of 3-dimethylamino-1-propylamino-DS-saponin (DMPS). The complete OVA cDNA was sub-cloned into a mammalian expression vector containing the human β-actin promotor and the neomycin resistant gene, under control of the SV40 promotor, to yield pAC-Neo-OVA. Negative control animals received PBS only. Animals were bled 14 days after the last immunization. Total IgG and IgG2a were determined by ELISA using OVA coated plates and a serum dilution of 1:100. The serum dilution was incubated at 37°C for 1 hour and the plates were washed. After incubation with anti IgG-HRP conjugate, the plates were washed and developed with a TMB substrate for 15 minutes at room temperature, stopped by the addition of 0.18 M sulfuric acid, and read at 450 nm.

Results

	Absorbance (450 nm)		
	PBS (-)	20 mg OVA DNA	20 mg OVA DNA +50 mg DMPS
1-	0.128	0.144	0.400
2-	0.089	0.080	0.210
3-	0.072	0.100	0.092
4-	0.084	0.096	0.140
5-	0.112	0.124	0.100
Average:	0.097 ± 0.018	0.109 ± 0.020	0.188 ± 0.093

[0145] The results, illustrated in FIGs. 1 and 2, demonstrate that the saponin derivatives of the present invention, when co-administered with a nucleic acid encoding for an antigen, stimulate the immune response in mice by stimulating antibody production.

[0146] Having now fully described this invention, it will be understood to those of ordinary skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations, and other parameters without affecting the scope of the invention or any embodiment thereof. All patents and publications cited herein are fully incorporated by reference herein in their entirety.

WHAT IS CLAIMED IS:

1. A saponin compound comprising:

(a) an aglycone core, wherein said aglycone core is covalently linked to one or more oligosaccharide chains; and

(b) a positively charged cationic chain, wherein said chain comprises

(i) three or more carbon atoms; and

(ii) one or more primary amine groups, one or more secondary amine groups, one or more tertiary amine groups, or one or more guanidine groups, or any combination thereof;

and wherein said positively charged cationic chain is covalently bound to said compound.

2. The compound of claim 1, further comprising:

(c) a lipophilic chain, wherein said lipophilic chain comprises from 4 to 36 carbon atoms and optionally contains one or more oxyethylene groups.

3. The compound of claim 1, wherein said aglycone core is a triterpenoid aglycone core.

4. The compound of 3, wherein said one or more oligosaccharide chains are covalently linked at C-3, at C-28, or at both C-3 and C-28, of said aglycone core.

5. The compound of claim 1, wherein said compound contains an aldehyde group or a ketone group that is capable of forming an imine or Schiff base with an amino group of a receptor protein on the surface of a cell.

6. The compound of claim 5, wherein said aldehyde or ketone group is attached to said aglycone core.

7. The compound of claim 5, wherein said aldehyde or ketone group is attached to one or more of said oligosaccharide chains.

8. The compound of claim 1, wherein said positively charged cationic chain is selected from the group consisting of a linear aliphatic chain, a branched aliphatic chain, an oligosaccharide, a polysaccharide, a protein, and a polypeptide that is cationic or has been subsequently modified by the introduction of amino groups or similar cationic basic groups that are capable of forming a complex with DNA or RNA.

9. The compound of claim 8, wherein said positively charged cationic chain is a protein.

10. The compound of claim 9, wherein said protein is a histone or protamine.

11. The compound of claim 1, wherein said cationic chain has a molecular weight ranging from 100 daltons to 100,000 daltons.

12. The compound of claim 1, wherein said cationic chain is attached either to a sugar residue of said oligosaccharide chain or to said aglycone core.

13. The compound of claim 2, wherein said lipophilic chain is attached to a sugar residue of said oligosaccharide chain or to said aglycone core.

14. A saponin derivative/polynucleotide complex formed by the association of a compound of claim 1 with a polynucleotide.

15. The complex of claim 14, wherein said polynucleotide is a DNA polynucleotide.

16. The complex of claim 15, wherein said DNA polynucleotide is a noncoding bacterial DNA polynucleotide.

17. The complex of claim 15, wherein said DNA polynucleotide encodes an immunogen.

18. A saponin derivative/polynucleotide secondary complex formed by association of the saponin derivative/polynucleotide complex of claim 14 with one or more saponins selected from the group consisting of a native saponin, a semi-synthetic saponin derivative, and a synthetic saponin containing a triterpenoid aglycone core covalently linked to one or more oligosaccharide chains.

19. A pharmaceutical composition comprising a compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

20. A pharmaceutical composition comprising:

(c) a compound of claim 1;

(d) a polynucleotide encoding an immunogen, wherein said polynucleotide is operably linked to a promoter; and

(e) a pharmaceutically acceptable carrier or diluent.

21. A pharmaceutical composition comprising:

(a) a compound of claim 1;

(b) a noncoding bacterial DNA polynucleotide; and

(c) a pharmaceutically acceptable carrier or diluent.

22. A pharmaceutical composition comprising a saponin derivative/polynucleotide complex of claims 14 or 15 and a pharmaceutically acceptable carrier or diluent.

23. A method of delivering a polynucleotide to cells of an animal in need thereof, comprising administration *in vivo* to the animal of a polynucleotide construct comprising a polynucleotide sequence encoding an immunogen, and a compound of claim 1.

24. The method of claim 23, wherein said polynucleotide construct forms a complex with said compound of claim 1.

25. The method of claim 23, wherein the animal is human.

26. The method of claim 23, wherein said polynucleotide sequence is a DNA sequence that is operably linked to a promoter.

27. The method of claim 23, wherein said polynucleotide sequence is mRNA.

28. The method of claim 23, wherein said construct is a plasmid DNA.

29. The method of claim 23, wherein said administration is intravenous, intramuscular, subcutaneous, transdermal, intranasal, or transmucosal.

30. A method of delivering a polynucleotide to cells of an animal in need thereof, comprising:

(a) forming a saponin derivative/nucleic acid complex, wherein said complex is formed by association of a compound of claim 1 and a polynucleotide sequence encoding an immunogen; and

(b) administering said complex in vitro to the cells of the animal in an amount sufficient that uptake of said polynucleotide sequence into the cells of the animal occurs.

31. A method of stimulating an immune response in an animal in need thereof, comprising

administering *in vivo* to the animal a noncoding bacterial DNA polynucleotide and a compound of claim 1.

32. The method of claim 31, wherein said noncoding bacterial DNA polynucleotide forms a complex with said compound of claim 1.

33. The method of claim 31, said method further comprising administering *in vivo* to the animal a polypeptide antigen.

34. The method of claim 31, said method further comprising administering *in vivo* to the animal a polynucleotide sequence encoding an immunogen.

35. A method of stimulating an immune response in an animal in need thereof, comprising

administering *in vivo* to the animal a polynucleotide sequence encoding an immunogen, wherein said polynucleotide sequence is operably linked to a promoter, and a compound of claim 1;

wherein said polynucleotide sequence is administered to the animal in an amount sufficient that uptake of said polynucleotide sequence into cells of the animal occurs, and sufficient expression results, to stimulate the immune response in the animal.

36. The method of claim 35, wherein said polynucleotide sequence forms a complex with said compound of claim 1.

37. A method of stimulating an immune response in an animal in need thereof, comprising:

(a) introducing into the cells of the animal a polynucleotide sequence encoding an immunogen, wherein said polynucleotide sequence is operably linked to a promoter, and a compound of claim 1; and

(b) introducing the cells into the animal, wherein sufficient expression of the immunogen occurs in the cells and an immune response is stimulated in the animal.

38. The method of claim 37, wherein said polynucleotide sequence forms a complex with said compound of claim 1.

39. A method of generating a detectable immune response in an animal in need thereof, comprising:

administering *in vivo* to the cells of an animal a polynucleotide sequence encoding an immunogen, wherein said polynucleotide sequence is operably linked to a promoter, and a compound of claim 1;

wherein said polynucleotide sequence is administered in an amount sufficient that uptake of said polynucleotide sequence into the cells of the animal occurs, and sufficient expression results, to generate the detectable immune response.

40. The method of claim 39, wherein said polynucleotide sequence forms a complex with said compound of claim 1.

41. A method of generating a detectable immune response in an animal in need thereof, comprising:

(a) introducing into the cells of the animal a polynucleotide sequence encoding an immunogen, wherein said polynucleotide sequence is operably linked to a promoter, and a compound of claim 1; and

(b) introducing the cells into the animal, wherein sufficient expression of the immunogen occurs in the cells and a detectable immune response if generated.

42. The method of claim 41, wherein said polynucleotide sequence forms a complex with said compound of claim 1.

FIG. 1

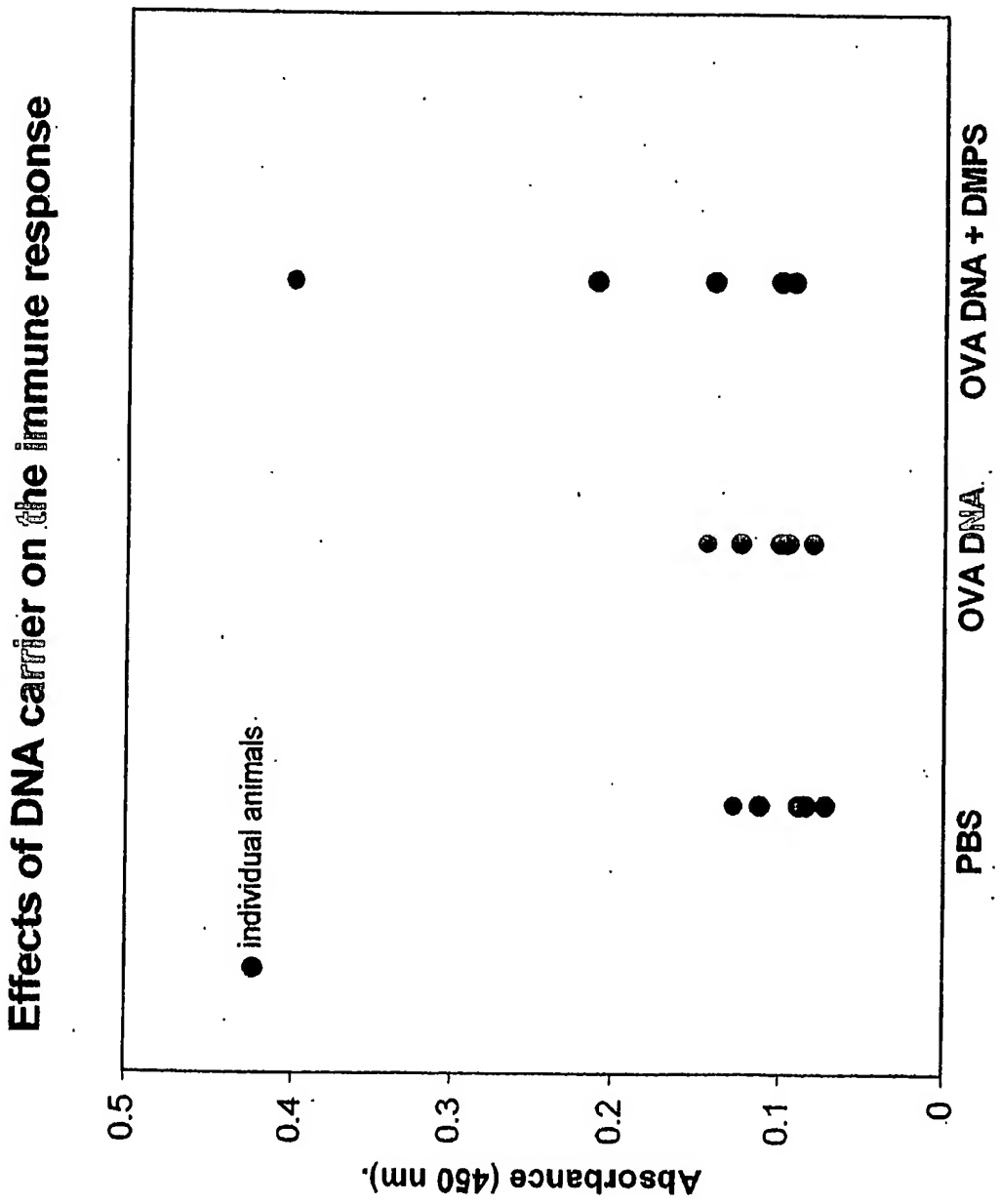

Effects of DNA carrier on the immune response
Absorbance (450 nm)
0.5
0.4
0.3
0.2
0.1
0
individual animals
PBS
OVA DNA
OVA DNA + DMPS

FIG. 2

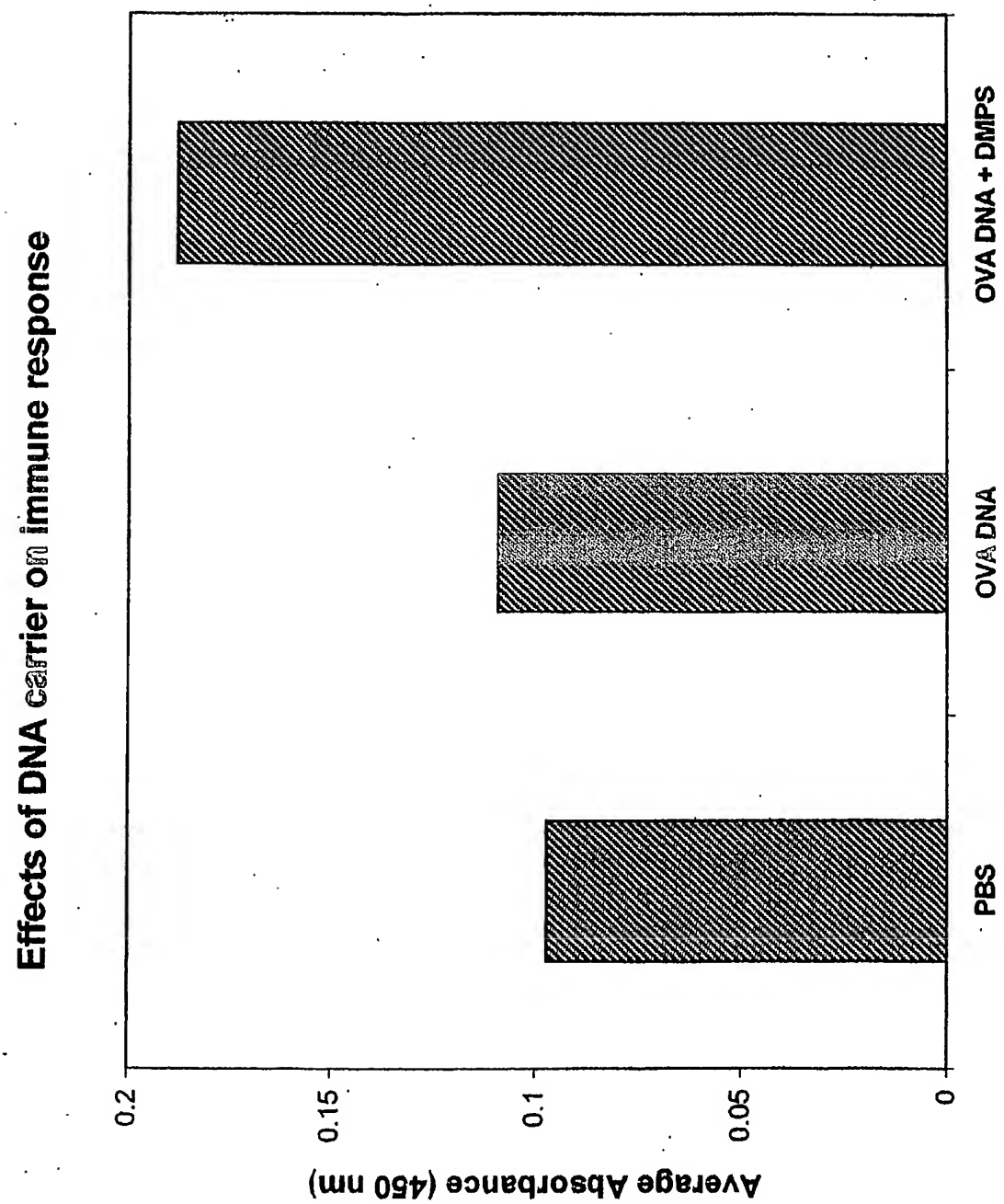
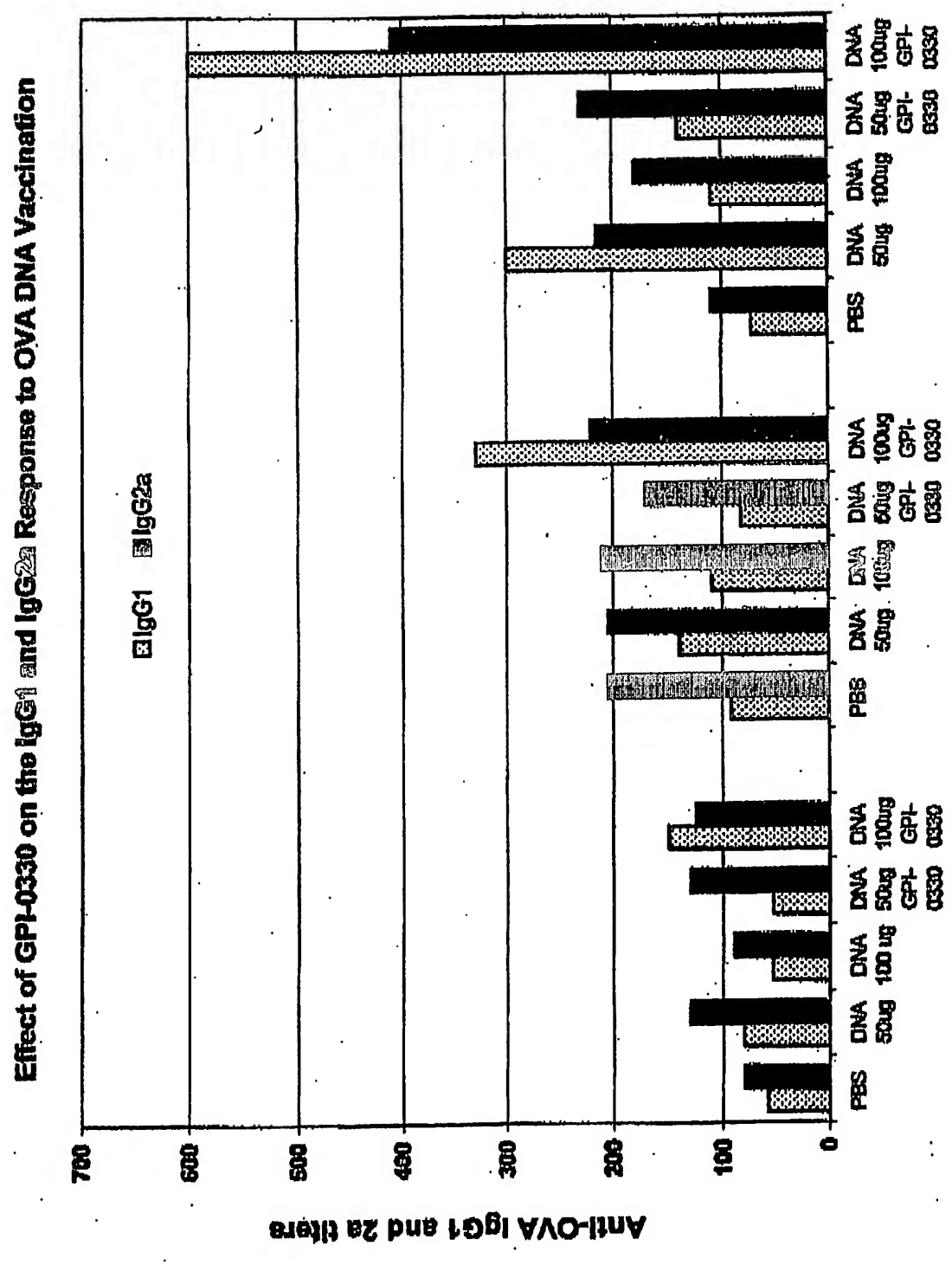

Effects of DNA carrier on immune response
Average Absorbance (450 nm)
0.2
0.15
0.1
0.05
0
PBS
OVA DNA
OVA DNA + DMPS

FIG. 3


Effect of GPI-0330 on the IgG1 and IgG2a Response to OVA DNA Vaccination
IgG1
IgG2a
Anti-OVA IgG1 and 2a titers
700
600
500
400
300
200
100
0
PBS
DNA 50ug
DNA 100 ug
DNA 50ug GPI-0330
DNA 100ug GPI-0330
PBS
DNA 50ug
DNA 100ug
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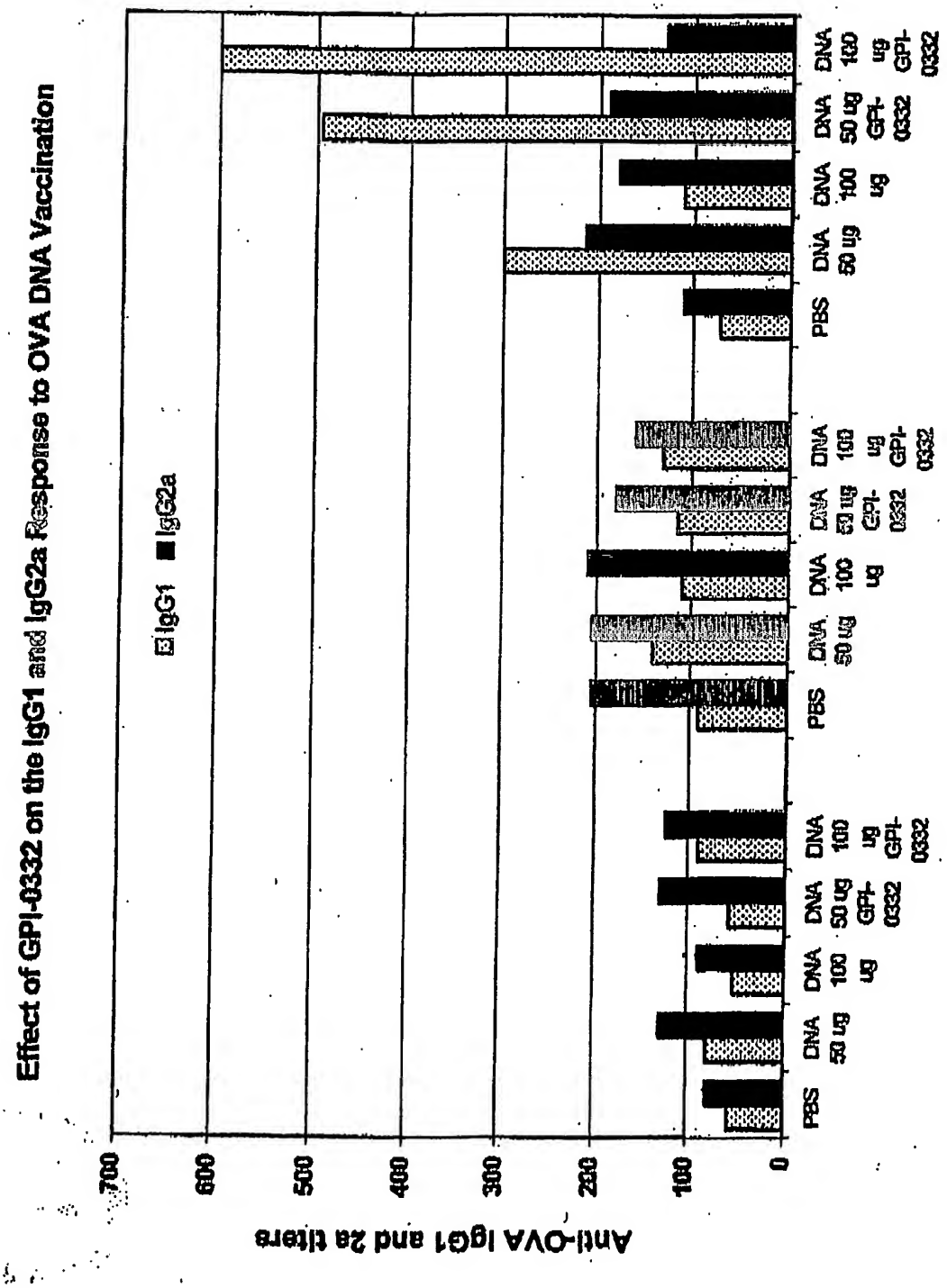
DNA 100ug
DNA 50ug GPI-0330
DNA 100ug GPI-0330

FIG. 4


Effect of GPI-0332 on the IgG1 and IgG2a Response to OVA DNA Vaccination
IgG1
IgG2a
Anti-OVA IgG1 and 2a titers
700
600
500
400
300
200
100
0
PBS
DNA 50 ug
DNA 100 ug
DNA 50 ug GPI-0332
DNA 100 ug GPI-0332

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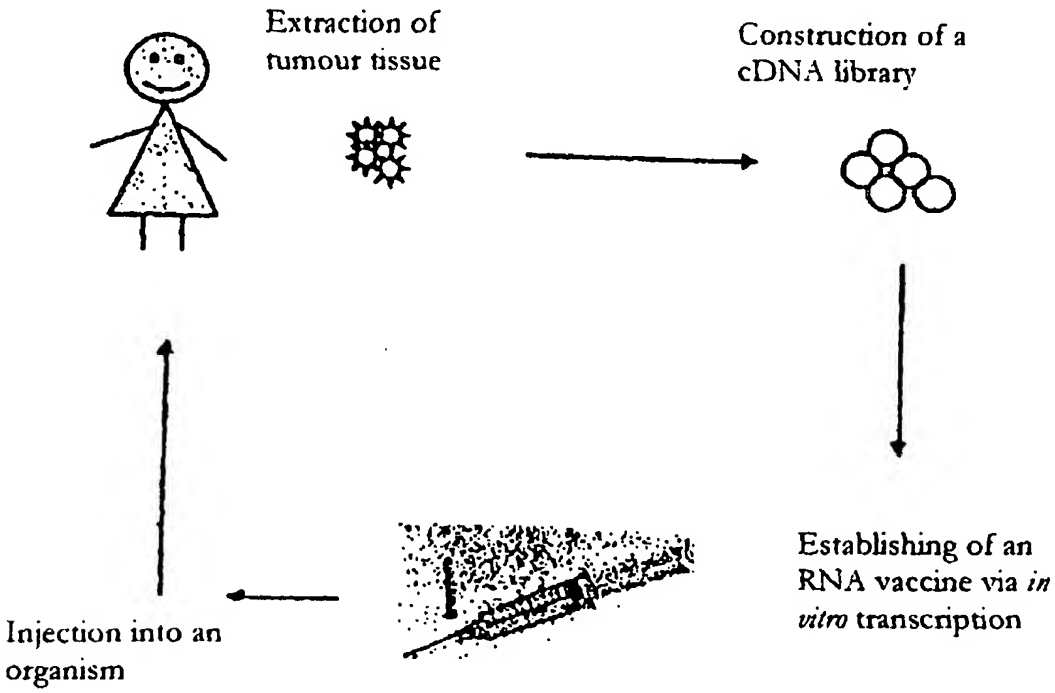
(54) Title: STABILISED MRNA TUMOUR VACCINE



(57) Abrégé/Abstract:
The invention relates to a pharmaceutical composition comprising at least one mRNA, which contains at least one region that codes for an antigen from a tumour, combined with an aqueous solvent and preferably with a cytokine, e.g. GM-CSF. The invention also relates to a method for producing the pharmaceutical composition. The inventive pharmaceutical composition is used in particular for the treatment and/or prophylaxis of cancer.

Abstract

The present invention relates to a pharmaceutical composition comprising at least one mRNA comprising at least one coding region for at least one antigen from a tumour, in combination with an aqueous solvent and preferably a cytokine, e.g. GM-CSF, and a process for the preparation of the pharmaceutical composition. The pharmaceutical composition according to the invention is used in particular for therapy and/or prophylaxis against cancer.

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CU01P009WO

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The application of mRNA for use as a therapeutic against tumour diseases

The present invention relates to a pharmaceutical composition comprising at least one mRNA comprising at least one coding region for at least one antigen from a tumour, in combination with an aqueous solvent and preferably a cytokine, e.g. GM-CSF, and a process for the preparation of the pharmaceutical composition. The pharmaceutical composition according to the invention is used in particular for therapy and/or prophylaxis against cancer.

Gene therapy and genetic vaccination are molecular medicine methods which, when used in the therapy and prevention of diseases, will have considerable effects on medical practice. Both methods are based on the introduction of nucleic acids into cells or into tissues of the patient and on subsequent processing of the information coded by the nucleic acids introduced, i.e. expression of the desired polypeptides.

The conventional procedure of methods of gene therapy and of genetic vaccination to date is the use of DNA to insert the required genetic information into the cell. Various methods for introducing DNA into cells have been developed in this connection, such as e.g. calcium phosphate

transfection, polyprene transfection, protoplast fusion, electroporation, microinjection and lipofection, whereas lipofection in particular having emerged as a suitable method.

A further method which has been proposed in particular in the case of genetic vaccination methods is the use of DNA viruses as DNA vehicles. Such viruses have the advantage that because of their infectious properties a very high transfection rate can be achieved. The viruses used are genetically modified, so that no functional infectious particles are formed in the transfected cell. In spite of this safety precaution, however, a certain risk of uncontrolled propagation of the genes having a gene therapy action and the viral genes introduced cannot be ruled out because of possible recombination events.

The DNA introduced into the cell is conventionally integrated into the genome of the transfected cell to a certain extent. On the one hand this phenomenon can exert a desired effect, since a long-lasting action of the DNA introduced can thereby be achieved. On the other hand, the integration into the genome results in a substantial risk of gene therapy. Thus e.g. the DNA introduced may be inserted into an intact gene, which represents a mutation which interferes or even completely switches off the function of the endogenous gene. On the one hand enzyme systems which are essential for the cell may be switched off by such integration events, and on the other hand there is also the danger of a transformation of the cell modified in this way into a degenerated state if a gene which is decisive for regulation of cell growth is modified by the

integration of the foreign DNA. A risk of the development of cancer therefore cannot be ruled out when using DNA viruses as gene therapeutics and vaccines. In this connection it is also to be noted that for effective expression of the genes introduced into the cell, the corresponding DNA vehicles contain a strong promoter, e.g. the viral CMV promoter. Integration of such promoters into the genome of the treated cell can lead to undesirable changes in the regulation of gene expression in the cell.

A further disadvantage of the use of DNA as gene therapeutics and vaccines is the induction of pathogenic anti-DNA antibodies in the patient, causing a possibly fatal immune response.

In contrast to DNA, the use of RNA as a gene therapeutic or vaccine is to be classified as substantially safer. In particular, RNA does not involve the risk of being integrated into the genome of the transfected cell in a stable manner. Furthermore, no viral sequences, such as promoters, are necessary for effective transcription. Moreover, RNA is degraded considerably more easily *in vivo*. Apparently because of the relatively short half-life of RNA in the blood circulation compared with DNA, no anti-RNA antibodies have been detected to date. RNA can therefore be regarded as the molecule of choice for molecular medicine therapy methods.

Nevertheless, medical methods based on RNA expression systems still require a solution to some fundamental problems before they are used more widely. One of the problems of using RNA is reliable cell- or tissue-specific

efficient transfer of the nucleic acid. Since RNA usually proves to be very unstable in solution, it has not hitherto been possible, or has been possible only in a very inefficient manner, to use RNA as a therapeutic or vaccine by the conventional methods which are used with DNA.

RNA-degrading enzymes, so-called RNAases (ribonucleases), are responsible for the instability. Even the smallest impurities of ribonucleases are sufficient to degrade RNA in solution completely. The natural degradation of mRNA in the cytoplasm of cells is very finely regulated. Several mechanisms are known in this respect. Thus, the terminal structure is of decisive importance for a functional mRNA. At the 5'-end is the so-called "cap structure" (a modified guanosine nucleotide), and at the 3'-end a sequence of up to 200 adenosine nucleotides (the so-called poly-A tail). The RNA is recognized as mRNA and the degradation is regulated via these structures. Moreover, there are further processes which stabilize or destabilize RNA. Many of these processes are still unknown, but an interaction between the RNA and proteins often appears to be decisive for this. For example, an "mRNA surveillance system" has recently been described (Hellerin and Parker, Annu. Rev. Genet. 1999, 33: 229 to 260), in which incomplete or nonsense mRNA is recognized by certain feedback protein interactions in the cytosol and is rendered accessible to degradation, the majority of these processes being performed by exonucleases.

Some measures for increasing the stability of RNA and thereby rendering possible its use as a gene therapeutic or RNA vaccine have been proposed in the prior art.

To solve the abovementioned problems of the instability of RNA *ex vivo*, EP-A-1083232 proposes a process for introduction of RNA, in particular mRNA, into cells and organisms, in which the RNA is in the form of a complex with a cationic peptide or protein.

WO 99/14346 describes further processes for stabilizing mRNA. In particular, modifications of the mRNA which stabilize the mRNA species against the degradation by RNases are proposed. Such modifications concern on the one hand stabilization by sequence modifications, in particular reduction of the C and/or U content by base elimination or base substitution. On the other hand, chemical modifications, in particular the use of nucleotide analogues, and 5'- and 3'-blocking groups, an increased length of the poly-A tail and complexing of the mRNA with stabilizing agents and combinations of the measures mentioned, are proposed.

The US patents US 5,580,859 and US 6,214,804 disclose, inter alia, mRNA vaccines and therapeutics in the context of "transient gene therapy" (TGT). Various measures for increasing the translation efficiency and the mRNA stability based above all on untranslated sequence regions are described.

Bieler and Wagner (in: Schleef (ed.), Plasmids for Therapy and Vaccination, chapter 9, pages 147 to 168, Wiley-VCH, Weinheim, 2001) report on the use of synthetic genes in connection with gene therapy methods using DNA vaccines and lentiviral vectors. The construction of a synthetic *gag*

gene derived from HIV-1, in which the codons were modified (alternative codon usage)compared with the wild-type sequence such that they corresponded to the use of codons which are to be found in highly expressed mammalian genes, is described. By this means, the A/T content in particular was reduced compared with the wild-type sequence. The authors find in particular an increased expression rate of the synthetic *gag* gene in transfected cells. Furthermore, in mice an increased formation of antibodies against the *gag* protein was observed in mice immunized with the synthetic DNA construct, and also an increased cytokine release *in vitro* in transfected spleen cells of mice. Finally, an induction of a cytotoxic immune response was to be found in mice immunized with the *gag* expression plasmid. The authors of this article attribute the improved properties of their DNA vaccine substantially to a change, caused by the optimized codon usage, to the nucleo-cytoplasmic transporation of the mRNA expressed by the DNA vaccine. In contrast, the authors consider the effect of the modified codon usage on the translation efficiency to be low.

The present invention is therefore based on the object of providing a new system for gene therapy and genetic vaccination for tumours which overcomes the disadvantages associated with the properties of DNA therapeutics and vaccines.

This object is solved by the embodiments of the present invention characterized in the claims.

In particular, a pharmaceutical composition comprising at least one mRNA comprising at least one coding region for at least one antigen from a tumour, in combination with an aqueous solvent, is provided.

According to the invention, the expression "antigen from a tumour" means that the corresponding antigen is expressed in cells associated with a tumour. According to the invention, antigens from tumours are therefore in particular those which are produced in the degenerated cells themselves. These are preferably antigens located on the surface of the cells. Furthermore, however, antigens from tumours are also those which are expressed in cells which are (were) not themselves (or originally themselves) degenerated but are associated with the tumour in question. These also include e.g. antigens which are connected with tumour-supplying vessels or (re)formation thereof, in particular those antigens which are associated with neovascularization or angiogenesis, e.g. growth factors, such as VEGF, bFGF etc. Such antigens connected with a tumour furthermore also include those from cells of the tissue embedding the tumour. Corresponding antigens of connective tissue cells, e.g. antigens of the extracellular matrix, are to be mentioned here.

According to the invention, in the pharmaceutical composition one (or more) mRNAs is used for therapy or inoculation, i.e. vaccination, for treatment or prevention (prophylaxis) of cancer diseases. The vaccination is based on the introduction of an antigen (or several antigens) of a tumour, in the present case the genetic information for the antigen in the form of the mRNA which codes for the

antigen(s), into the organism, in particular into the cell. The mRNA contained in the pharmaceutical composition is translated into the (tumour) antigen, i.e. the polypeptide or antigenic peptide coded by the modified mRNA is expressed, as a result of which an immune response directed against this polypeptide or antigenic polypeptide is stimulated. In the present case of the use as genetic vaccines for treatment of cancer, the immune response is therefore achieved by introduction of the genetic information for antigens from a tumour, in particular proteins which are expressed exclusively on cancer cells, in that a pharmaceutical composition according to the invention which comprises an mRNA which codes for such a cancer antigen is administered. By this means, the cancer antigen(s) is (are) expressed in the organism, as a result of which an immune response which is directed effectively against the cancer cells is provoked.

In its use as a vaccine, the pharmaceutical composition according to the invention is to be considered in particular for treatment of cancer diseases (the mRNA preferably coding for a tumour-specific surface antigen (TSSA), e.g. for treatment of malignant melanoma, colon carcinoma, lymphomas, sarcomas, small-cell pulmonary carcinoma, blastomas etc. Specific examples of tumour antigens are, inter alia, 707-AP, AFP, ART-4, BAGE, β-catenine/m, Bcr-abl, CAMEL, CAP-1, CASP-8, CDC27/m, CDK4/m, CEA, CT, Cyp-B, DAM, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, Gp100, HAGE, HER-2/neu, HLA-A*0201-R170I, HPV-E7, HSP70-2M, HAST-2, hTERT (or hTRT), iCE, KIAA0205, LAGE, LDLR/FUT, MAGE, MART-1/melan-A, MC1R, myosine/m, MUC1, MUM-1, -2, -3, NA88-A, NY-ESO-1, p190 minor bcr-abl, Pml/RARα, PRAME, PSA,

PSM, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, TEL/AML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2 and WT1.

According to a further preferred embodiment, the antigen(s) from a tumour is or are a polyepitope of the antigen(s) from a tumour. A "polyepitope" of an antigen or several antigens is an amino acid sequence in which several or many regions of the antigen(s) which interact with the antigen-binding part of an antibody or with a T cell receptor are represented. In this context, the polyepitope can be complete and non-modified. However, according to the present invention it can also be modified, in particular to optimize the antibody/antigen and T cell receptor/antigen interaction, respectively. A modification compared with the wild-type polyepitope can include e.g. a deletion, addition and/or substitution of one or more amino acid residues. Accordingly, in the mRNA of the present invention which codes for the modified polyepitope, one or more nucleotides is/are removed, added and/or replaced, compared with the mRNA which codes for the wild-type polyepitope.

In order to increase the stability of the (m)RNA contained in the pharmaceutical composition of the present invention, each (m)RNA contained in the pharmaceutical composition preferably has one or more modifications, in particular chemical modifications, which contribute towards increasing the half-life of the (m)RNA (one or more) in the organism or improve the transfer of the (m)RNA (one or more) into the cell.

For example, in the sequences of eukaryotic mRNAs, there are destabilizing sequence elements (DSE) to which signal

proteins bind and regulate the enzymatic degradation of the mRNA *in vivo*. For further stabilization of the modified mRNA preferably contained in the pharmaceutical composition according to the invention, where appropriate in the region which codes for at least one antigen from a tumour one or more modifications compared with the corresponding region of the wild-type mRNA are carried out, so that no destabilizing sequence elements are present. According to the invention, it is of course also preferable, where appropriate, to eliminate from the mRNA DSEs present in the untranslated regions (3'- and/or 5'-UTR).

Such destabilizing sequences are e.g. AU-rich sequences ("AURES"), which occur in 3'-UTR sections of numerous unstable mRNAs (Caput et al., Proc. Natl. Acad. Sci. USA 1986, 83: 1670 to 1674). The RNA molecules contained in the pharmaceutical composition according to the invention are therefore preferably modified compared with the wild-type mRNA such that they contain no such destabilizing sequences. This also applies to those sequence motifs which are recognized by possible endonucleases, e.g. the sequence GAACAAG, which is contained in the 3'-UTR segment of the gene which codes for the transferrin receptor (Binder et al., EMBO J. 1994, 13: 1969 to 1980). These sequence motifs are also preferably eliminated in the modified mRNA of the pharmaceutical composition according to the invention.

A skilled person in the art is familiar with various processes which are suitable for substitution of codons in the modified mRNA according to the invention. In the case of relatively short coding regions (which code for biologically active or antigenic peptides) e.g. the total

mRNA can be synthesized chemically using standard techniques.

Nevertheless, base substitutions are preferably introduced, using a DNA matrix for the preparation of the modified mRNA with the aid of techniques of the usual targeted mutagenesis; Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 3rd ed., Cold Spring Harbor, NY, 2001.

In this process, for the preparation of the mRNA, a corresponding DNA molecule is therefore transcribed *in vitro*. This DNA matrix has a suitable promoter, e.g. a T7 or SP6 promoter, for the *in vitro* transcription, which is followed by the desired nucleotide sequence for the mRNA to be prepared and a termination signal for the *in vitro* transcription. According to the invention, the DNA molecule which forms the matrix of the RNA construct to be prepared is prepared by fermentative proliferation and subsequent isolation as part of a plasmid which can be replicated in bacteria. Plasmids which may be mentioned as suitable for the present invention are e.g. the plasmids pT7TS (GenBank Access Number U26404; Lai et al., Development 1995, 121: 2349 to 2360; cf. also fig. 8), pGEM® serie, e.g. pGEM®-1 (GenBank Access Number X65300; from Promega) and pSP64 (GenBank Access Number X65327); cf. also Mezei and Storts, Purification of PCR Products, in: Griffin and Griffin (ed.), PCR Technology: Current Innovation, CRC Press, Boca Raton, FL, 2001.

Using short synthetic DNA oligonucleotides which contain short single-stranded transitions at the cleavage sites

formed or genes prepared by chemical synthesis, the desired nucleotide sequence can thus be cloned into a suitable plasmid by molecular biology methods with which a skilled person in the art is familiar (cf. Maniatis et al., see above). The DNA molecule is then excised the plasmid, in which it can be present in one or multiple copy, by digestion with restriction endonucleases.

The modified mRNA contained in the pharmaceutical composition according to the invention can moreover have a 5'-cap structure (a modified guanosine nucleotide). Examples of cap structures which may be mentioned are m7G(5')ppp (5'(A,G(5')ppp(5')A and G(5')ppp(5')G.

According to a further preferred embodiment of the present invention, the modified mRNA contains a poly(A^+) tail of at least about 25, in particular at least about 30, preferably at least about 50 nucleotides, more preferably at least about 70 nucleotides, particularly preferably at least about 100 nucleotides. However, the poly(A^+) tail can also comprise 200 and more nucleotides.

For efficient translation of the mRNA, effective binding of the ribosomes to the ribosome binding site (Kozak sequence: GCCGCCACC**AUG**G, **AUG** forms the start codon) is necessary. In this respect, it has been found that an increased A/U content around this site renders possible a more efficient ribosome binding to the mRNA.

It is furthermore possible to insert one or more so-called IRES ("internal ribosomal entry site) into the mRNA. An IRES can thus function as the single ribosome binding site,

but it can also serve to provide an mRNA which codes several peptides or polypeptides which are to be translated by the ribosomes independently of one another ("multicistronic" or "polycistronic" mRNA). Examples of IRES sequences which can be used according to the invention are those from picornaviruses (e.g. FMDV), pestviruses (CFFV), polioviruses (PV), encephalomyocarditis viruses (ECMV), foot and mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical swine fever viruses (CSFV), mouse leukoma virus (MLV), simian immunodeficiency viruses (SIV) or cricket paralysis viruses (CrPV).

According to a further preferred embodiment of the present invention, the mRNA has, in the 5'-and/or 3'-untranslated regions, stabilizing sequences which are capable of increasing the half-life of the mRNA in the cytosol.

These stabilizing sequences can have a 100 % sequence homology to naturally occurring sequences which occur in viruses, bacteria and eukaryotes, but can also be partly or completely of synthetic nature. Examples of stabilizing sequences which can be used in the present invention and which may be mentioned are the untranslated sequences (UTR) of the β-globin gene, e.g. from *Homo sapiens* or *Xenopus laevis*. Another example of a stabilizing sequence has the general formula $(C/U)CCAN_xCCC(U/A)Py_xUC(C/U)CC$, which is contained in the 3'-UTR of the very stable mRNA which codes for α-globin, α-(I)-collagen, 15-lipoxygenase or for tyrosine hydroxylase (cf. Holcik et al., Proc. Natl. Acad. Sci. USA 1997, 94: 2410 to 2414). Such stabilizing sequences can of course be used individually or in combination with one another and also in combination with

other stabilizing sequences known to a skilled person in the art.

For further stabilization of the mRNA, it is moreover preferred to contain at least one analogue of naturally occurring nucleotides. This is based on the fact that the RNA-degrading enzymes occurring in the cells preferentially recognize naturally occurring nucleotides as a substrate. The degradation of RNA can therefore be made difficult by insertion of nucleotide analogues, whereby the effect on the translation efficiency on insertion of these analogues, in particular in the coding region of the mRNA, can have a positive or negative effect on the translation efficiency.

In a list which is in no way conclusive, examples which may be mentioned of nucleotide analogues which can be used according to the invention are phosphoroamidates, phosphorothioates, peptide nucleotides, methylphosphonates, 7-deazaguanosine, 5-methylcytosine and inosine. The preparation of such analogues is known to a skilled person in the art e.g. from the US patents 4,373,071, US 4,401,796, US 4,415,732, US 4,458,066, US 4,500,707, US 4,668,777, US 4,973,679, US 5,047,524, US 5,132,418, US 5,153,319, US 5,262,530 and 5,700,642. According to the invention, such analogues can occur in untranslated and translated regions of the modified mRNA.

Furthermore, effective transfer of the preferably modified mRNA into the cells to be treated or the organism to be treated can be improved if the mRNA is associated with a cationic or polycationic agent, in particular a corresponding peptide or protein, or bound thereto. The

mRNA is therefore present in the pharmaceutical composition according to the invention preferably in a form complexed or condensed with such an agent. In particular, the use of protamine as a polycationic, nucleic acid-binding protein is particularly effective in this context. The use of other cationic peptides or proteins, such as poly-L-lysine, poly-L-arginine or histones, is furthermore also possible. This procedure for stabilizing the modified mRNA is described in EP-A-1083232, the disclosure content of which in this respect is included in its full scope in the present invention.

The mRNA modified according to the invention can moreover also contain, in addition to the peptide or polypeptide which is antigenic or active in gene therapy, at least one further functional section which e.g. codes for a cytokine which promotes the immune response, (monokine, lymphokine, interleukin or chemokine, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IFN-α, IFN-γ, GM-CFS, LT-α or growth factors, such as hGH).

The pharmaceutical composition according to the invention can further comprise one or more adjuvants to increase the immunogenicity. "Adjuvant" here is to be understood as meaning any chemical or biological compound which promotes a specific immune response. Various mechanisms are possible in this respect, depending on the various types of adjuvants used. For example, compounds which promote endocytosis of the modified mRNA contained in the pharmaceutical composition by dendritic cells (DC) form a first class of adjuvants which can be used. Other compounds which allow the maturation of the DC, e.g.

lipopolysaccharides, TNF-α or CD40 ligand, are a further class of suitable adjuvants. Generally, any agent which influences the immune system of the nature of a "warning signal" (LPS, GP96, oligonucleotides with the CpG motif) or cytokines, in particular GM-CSF, can be used as an adjuvant which allow an immune response against an antigen which is coded by the modified mRNA to be increased and/or influenced in a targeted manner. In particular, the abovementioned cytokines are preferred in this context. Further known adjuvants are aluminium hydroxide, Freund's adjuvant and the abovementioned stabilizing cationic peptides or polypeptides, such as protamine. Lipopeptides, such as Pam3Cys, are also particularly suitable for use as adjuvants in the pharmaceutical composition of the present invention; c.f. Deres et al., Nature 1989, 342: 561-564.

Further particularly suitable adjuvants are moreover (other) RNA or also mRNA species, which can be added to the pharmaceutical composition of the present invention to increase the immunogenicity. Such adjuvant RNA is advantageously chemically modified for stabilization ("cis modification" or "cis stabilization"), for example by the abovementioned nucleotide analogues, in particular phosphorothioate-modified nucleotides, or by the above further measures for stabilization of RNA. A further advantageous possibility of stabilization is complexing or association ("trans association" or "trans modification" and "trans stabilization", respectively) with the abovementioned cationic or polycationic agents, e.g. with protamine.

According to a further advantageous embodiment, the stability of the RNA molecules contained in the pharmaceutical composition (mRNA, coding for a tumour antigen, and optionally adjuvant (m)RNA) is increased by one or more RNase inhibitors. Preferred RNase inhibitors are peptides or proteins, in particular those from the placenta (e.g. from the human placenta) or pancreas. Such RNase inhibitors can also be in a recombinant form. A specific example of an RNase inhibitor is RNasin®, which is commercially obtainable, e.g. from Promega. Such RNase inhibitors can be used generally for stabilizing RNA. A pharmaceutical composition comprising at least one RNA, in particular mRNA, which codes for at least one antigen, and at least one RNase inhibitor as defined above, optionally in combination with a pharmaceutically acceptable solvent, carrier and/or vehicle, is therefore also provided generally according to the invention. Corresponding antigens in a general form and solvents, carriers and vehicles are defined below. In respect of preferred tumour antigens, reference is made to the statements in this respect concerning the preferred pharmaceutical composition comprising at least one mRNA which codes for at least one antigen from a tumour.

The pharmaceutical composition according to the invention preferably comprises, in addition to the aqueous solvent and the mRNA, one or more further pharmaceutically acceptable carrier(s) and/or one or more further pharmaceutically acceptable vehicle(s). Corresponding routes for suitable formulation and preparation of the pharmaceutical composition according to the invention are disclosed in "Remington's Pharmaceutical Sciences" (Mack

Pub. Co., Easton, PA, 1980), which is a constituent in its full content of the disclosure of the present invention. Possible carrier substances for parenteral administration are e.g., in addition to sterile water or sterile saline solutions as aqueous solvents, also polyalkylene glycols, hydrogenated naphthalene and, in particular, biocompatible lactide polymers, lactide/glycolide copolymers or polyoxyethylene/polyoxypropylene copolymers. Compositions according to the invention can comprise filler substances or substances such as lactose, mannitol, substances for covalent linking of polymers, such as e.g. polyethylene glycol, to inhibitors according to the invention, complexing with metal ions or inclusion of materials in or on particular preparations of a polymer compound, such as e.g. polylactate, polyglycolic acid or hydrogel, or on liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte fragments or spheroplasts. The particular embodiments of the compositions are chosen according to the physical properties, for example in respect of solubility, stability, bioavailability or degradability. Controlled or constant release of the active compound component according to the invention in the composition includes formulations based on lipophilic depots (e.g. fatty acids, waxes or oils). Coatings of substances according to the invention or compositions comprising such substances, that is to say coatings with polymers (e.g. polyoxamers or polyoxamines) are also disclosed in the context of the present invention. Substances or compositions according to the invention can furthermore have protective coatings, e.g. protease inhibitors or permeability-increasing agents. Preferred aqueous carrier materials are e.g. water for

injection (WFI) or water buffered with phosphate, citrate or acetate etc., whereby the pH typically being adjusted to 5.0 to 8.0, preferably 6.0 to 7.0. The aqueous solvent or the further carrier(s) or the further vehicle(s) will additionally preferably comprise salt constituents, e.g. sodium chloride, potassium chloride or other components which render the solution e.g. isotonic. Aqueous solvents or the further carrier(s) or the further vehicle(s) can furthermore comprise, in addition to the abovementioned constituents, additional components, such as human serum albumin (HSA), Polysorbate 80, sugars or amino acids.

The method and mode of administration and the dosage of the pharmaceutical composition according to the invention depend on the disease to be treated and the stage of advancement thereof, and also the body weight, the age and the sex of the patient.

The concentration of the modified mRNA in such formulations can therefore vary within a wide range from 1 µg to 100 mg/ml. The pharmaceutical composition according to the invention is preferably administered to the patient parenterally, e.g. intravenously, intraarterially, subcutaneously or intramuscularly. It is also possible to administer the pharmaceutical composition topically or orally. The pharmaceutical composition according to the invention is preferably administered intradermally. A transdermal administration with the aid of electric currents or by osmotic forces is furthermore possible. The pharmaceutical composition of the present invention can moreover be injected locally into a tumour.

Thus, a method for treatment or a vaccination method for prevention of cancer diseases or the abovementioned diseases which comprises administration of the pharmaceutical composition according to the invention to a patient, in particular a human, is thus also provided according to the invention.

According to a preferred embodiment of the treatment or vaccination method or in the use, defined above, of the mRNA according to the invention which codes for at least one antigen from a tumour for the preparation of a pharmaceutical composition for treatment and/or prevention of cancer diseases one or more cytokine(s) is administered to the patient, in addition to the pharmaceutical composition according to the invention.

A treatment or vaccination method comprising administration of at least one RNA, preferably mRNA, which code(s) for at least one antigen from a tumour (in accordance with the above definition) and is (are) optionally stabilized in accordance with the above statements, and at least one cytokine, e.g. one or more of the abovementioned cytokines, in particular GM-CSF, to a patient, in particular a human, is therefore also provided generally according to the invention. The method is used in particular for treatment and/or prevention of corresponding cancer diseases (e.g. the above cancer diseases). The present invention is accordingly also directed generally to a pharmaceutical composition comprising at least one RNA, preferably mRNA, which code(s) for at least one antigen from a tumour (according to the above definition) and is (are) optionally stabilized in accordance with the above statements, and at

least one cytokine, e.g. one or more of the abovementioned cytokines, such as GM-CSF, preferably in combination with a pharmaceutically acceptable carrier and/or vehicle, e.g. an aqueous solvent, or one or more of the carriers, solvents or vehicles defined above. The use of cytokines, e.g. one or more of the abovementioned cytokines, in particular GM-CSF, in combination with one or more RNA molecule(s) as defined above, for treatment and/or prevention of cancer diseases (e.g. cancer diseases listed above) is thus also disclosed according to the invention.

According to a further preferred embodiment of the present invention, the cytokine, e.g. GM-CSF, is administered simultaneously with or, which is more preferable, before or after the pharmaceutical composition comprising the mRNA which codes for at least one antigen from a tumour (or is used for the preparation of a corresponding medicament for simultaneous administration with or for administration before or after the abovementioned (m)RNA). The administration of the cytokine, in particular GM-CSF, is very particularly preferably carried out shortly before (e.g. about 15 min or less, e.g. about 10 or about 5 min) or a relatively short time (e.g. about 5, 10, 15, 30, 45 or 60 min) after or a longer time (e.g. about 2, 6, 12, 24 or 36 h) after the administration of the pharmaceutical composition defined above or generally after the (m)RNA of at least one which codes for at least one antigen from a tumour.

The application of the cytokine, e.g. GM-CSF, can be carried out in this context by the same route as the pharmaceutical composition according to the invention or

the at least one (m)RNA which codes for at least one antigen from a tumour or in a manner separate from this. Suitable administration routes and also the suitable formulation possibilities in respect of the cytokine(s) can be found from the above statements in respect of the pharmaceutical compositions according to the invention. In the case of a human patient, a GM-CSF dose of 100 micrograms/m^2 in particular is advisable. The administration of the cytokine, e.g. GM-CSF, is particularly preferably carried out by an s.c. injection.

The pharmaceutical compositions of the present invention or the RNA which codes for an antigen from a tumour and where appropriate, in association therewith, the cytokine(s) are preferably administered in the form of interval doses. For example, a dose of a pharmaceutical composition according to the invention can be administered in relatively short intervals, e.g. daily, every second day, every third day etc., or, which is more preferable, in longer intervals, e.g. once weekly, once in two weeks, once in three weeks, once a month etc. The intervals can also be changeble in this context, whereby it being necessary in particular to take into account the immunological parameters of the patient. For example, the administration of a pharmaceutical composition according to the invention (and where appropriate, in association therewith, also the administration of the cytokine(s)) can follow a treatment plan in which the interval is shorter, e.g. once in two weeks, at the start of the treatment and then, depending on the course of treatment or the appropriately determined immunological parameters of the patient, the interval is lengthened to e.g. once a month. A therapy plan tailor-made

to the particular individual can thus be applied according to the patient, in particular his condition and his immunological parameters.

The present invention also provides a process for the preparation of the pharmaceutical composition defined above, comprising the steps:

(a) preparation of a cDNA library, or a part thereof, from tumour tissue of a patient,

(b) preparation of a matrix for *in vitro* transcription of RNA with the aid of the cDNA library or a part thereof and

(c) *in vitro* transcribing of the matrix.

The tumour tissue of the patient can be obtained e.g. by a simple biopsy. However, it can also be provided by surgical removal of tumour-invaded tissue. The preparation of the cDNA library or a part thereof according to step (a) of the preparation process of the present invention can moreover be carried out after the corresponding tissue has been deep-frozen for storage, preferably at temperatures below -70°C. For preparation of the cDNA library or a part thereof, isolation of the total RNA, e.g. from a tumour tissue biopsy, is first carried out. Processes for this are described e.g. in Maniatis et al., *supra*. Corresponding kits are furthermore commercially obtainable for this, e.g. from Roche AG (e.g. the product "High Pure RNA Isolation Kit"). The corresponding poly(A$^+$) RNA is isolated from the total RNA in accordance with processes known to a person skilled in the art (cf. e.g. Maniatis et al., *supra*). Appropriate kits are also commercially obtainable for this. An example is the "High Pure RNA Tissue Kit" from Roche AG.

Starting from the poly(A^+) RNA obtained in this way, the cDNA library is then prepared (in this context cf. also e.g. Maniatis et al., *supra*). For this step in the preparation of the cDNA library also, commercially obtainable kits are available to a person skilled in the art, e.g. the "SMART PCR cDNA Synthesis Kit" from Clontech Inc. The individual sub-steps from the poly(A^+) RNA to the double-stranded cDNA is shown schematically in fig. 11 by the example of the process in accordance with the "SMART PCR cDNA Synthesis Kit" from Clontech Inc.

According to step (b) of the above preparation process, starting from the cDNA library (or a part thereof), a matrix is synthesized for the *in vitro* transcription. According to the invention, this is effected in particular by cloning the cDNA fragments obtained into a suitable RNA production vector. The suitable DNA matrix and the plasmids which are preferred according to the invention are already mentioned above in connection with the preparation of the mRNA for the pharmaceutical composition according to the invention.

For *in vitro* transcription of the matrix prepared in step (b) according to the invention, these are first linearized with a corresponding restriction enzyme, if they are present as circular plasmid (c)DNA. Preferably, the construct cleaved in this way is purified once more, e.g. by appropriate phenol/chloroform and/or chloroform/phenol/isoamyl alcohol mixtures, before the actual *in vitro* transcription. By this means it is ensured in particular that the DNA matrix is in a protein-free form. The enzymatic synthesis of the RNA is then carried

out starting from the purified matrix. This sub-step takes place in an appropriate reaction mixture comprising the linearized, protein-free DNA matrix in a suitable buffer, to which a ribonuclease inhibitor is preferably added, using a mixture of the required ribonucleotide triphosphates (rATP, rCTP, rUTP and rGTP) and a sufficient amount of a RNA polymerase, e.g. T7 polymerase. The reaction mixture is present here in RNase-free water. Preferably, a CAP analogue is also added during the actual enzymatic synthesis of the RNA. After an incubation of an appropriately long period, e.g. 2 h, at 37 °C, the DNA matrix is degraded by addition of RNase-free DNase, incubation preferably being carried out again at 37 °C.

Preferably, the RNA prepared in this way is precipitated by means of ammonium acetate/ethanol and, where appropriate, washed once or several times with RNase-free ethanol. Finally, the RNA purified in this way is dried and, according to a preferred embodiment, is taken up in RNase-free water. The RNA prepared in this way can moreover be subjected to several extractions with phenol/chloroform or phenol/chloroform/isoamyl alcohol.

According to a further preferred embodiment of the preparation process defined above, only a part of a total cDNA library is obtained and converted into corresponding mRNA molecules. According to the invention, a so-called subtraction library can therefore also be used as part of the total cDNA library in order to provide the mRNA molecules according to the invention. A preferred part of the cDNA library of the tumour tissue codes for the tumour-specific antigens. For certain tumours, the corresponding

antigens are known. According to a further preferred embodiment, the part of the cDNA library which codes for the tumour-specific antigens can first be defined (i.e. before step (a) of the process defined above). This is preferably effected by determining the sequences of the tumour-specific antigens by an alignment with a corresponding cDNA library from healthy tissue.

The alignment according to the invention comprises in particular a comparison of the expression pattern of the healthy tissue with that of the tumour tissue in question. Corresponding expression patterns can be determined at the nucleic acid level e.g. with the aid of suitable hybridization experiments. For this e.g. the corresponding (m)RNA or cDNA libraries of the tissue can in each case be separated in suitable agarose or polyacrylamide gels, transferred to membranes and hybridized with corresponding nucleic acid probes, preferably oligonucleotide probes, which represent the particular genes (northern and southern blots, respectively). A comparison of the corresponding hybridizations thus provides those genes which are expressed either exclusively by the tumour tissue or to a greater extent therein.

According to a further preferred embodiment, the hybridization experiments mentioned are carried out with the aid of a diagnosis by microarrays (one or more microarrays). A corresponding DNA microarray comprises a defined arrangement, in particular in a small or very small space, of nucleic acid, in particular oligonucleotide, probes, each probe representing e.g. in each case a gene, the presence or absence of which is to be investigated in

the corresponding (m)RNA or cDNA library. In an appropriate microarrangement, hundreds, thousands and even tens to hundreds of thousands of genes can be represented in this way. For analysis of the expression pattern of the particular tissue, either the poly(A$^+$) RNA or, which is preferable, the corresponding cDNA is then marked with a suitable marker, in particular fluorescence markers are used for this purpose, and brought into contact with the microarray under suitable hybridization conditions. If a cDNA species binds to a probe molecule present on the microarray, in particular an oligonucleotide probe molecule, a more or less pronounced fluorescence signal, which can be measured with a suitable detection apparatus, e.g. an appropriately designed fluorescence spectrometer, is accordingly observed. The more the cDNA (or RNA) species is represented in the library, the greater will be the signal, e.g. the fluorescence signal. The corresponding microarray hybridization experiment (or several or many of these) is (are) carried out separately for the tumour tissue and the healthy tissue. The genes expressed exclusively or to an increased extent by the tumour tissue can therefore be concluded from the difference between the signals read from the microarray experiments. Such DNA microarray analyses are described e.g. in Schena (2002), Microarray Analysis, ISBN 0-471-41443-3, John Wiley & Sons, Inc., New York, the disclosure content in this respect of this document being included in its full scope in the present invention.

However, the establishing of tumour tissue-specific expression patterns is in no way limited to analyses at the nucleic acid level. Methods known from the prior art which

serve for expression analysis at the protein level are of course also familiar to a person skilled in the art. There may be mentioned here in particular techniques of 2D gel electrophoresis and mass spectrometry, whereby these techniques advantageously also can be combined with protein biochips (i.e., microarrays at the protein level, in which e.g. a protein extract from healthy or tumour tissue is brought into contact with antibodies and/or peptides applied to the microarray substrate). With regard to the mass spectroscopy methods, MALDI-TOF ("matrix assisted laser desorption/ionization-time of flight") methods are to be mentioned in this respect. The techniques mentioned for protein chemistry analysis to obtain the expression pattern of tumour tissue in comparison with healthy tissue are described e.g. in Rehm (2000) Der Experimentator: Proteinbiochemie/Proteomics [The Experimenter: Protein Biochemistry/Proteomics], Spektrum Akademischer Verlag, Heidelberg, 3rd ed., to the disclosure content of which in this respect reference is expressly made *expressis verbis* in the present invention. With regard to protein microarrays, reference is moreover again made to the statements in this respect in Schena (2002), *supra*.

The figures show:

Fig. 1 shows a graphical view of the results of a tumour vaccination, with RNA, of mice (rat Her-2/neu transgenic animals) which develop mammary carcinomas spontaneously. The tumour multiplicity is plotted on the y-axis against the age of the mice on the x-axis. Untreated mice (n = 4), which served as a control, all had tumours at an age of 6

months. Three mice were injected with DNA which codes for Her-2/neu, one mouse being tumour-free after 10 months. As a further negative control, 4 mice received an antisense mRNA complementary to the mRNA for Her-2/neu. These mice also all had tumours after 6 months (not shown). In contrast, one of 4 mice which were injected with mRNA which codes for Her-2/neu (i.e., the sense strand) was tumour-free after 9 months.

Fig. 2 shows a graphical view of the results of experiments relating to beta-galactosidase (beta-Gal)-specific CTL (cytotoxic T lymphocyte) activity by immunization with an mRNA which codes for beta-Gal, under the influence of GM-CSF. BALB/c mice were immunized with 25 µg of mRNA which codes for beta-Gal by injection into the inner auricula. The splenocytes were stimulated with beta-Gal protein *in vitro* and the CTL activity was determined 6 days after the *in vitro* stimulation using a standard ^{51}Cr release test. The target cells were P815 (H_2^d) cells which were charged (■) with the synthetic peptide TPHPARIGL, which corresponds to the H_2^d epitope of beta-Gal, or were not charged (▲). In each case three or two animals were treated per group. Animals which were injected i.d. in both auriculae with only injection buffer served as a negative control. Animals which were injected i.d. in both auriculae with 10 µg of a plasmid which codes for beta-Gal in PBS served as a positive control ("DNA"). The test groups received RNA which codes for beta-Gal by itself or in combination with

GM-CSF, which was injected 24 h ("GM-CSF t-1"), 2 h before the RNA injection ("GM-CSF t0") or 24 h after the RNA injection ("GM-CSF t+1") into the same site (into the auriculae) or at another site (s.c. on the back). In each case three different effector/target cell ratios (200, 44, 10) were tested.

Fig. 3 shows further graphical views of the results of ELISA standard tests specific for IFN-gamma (A) and IL-4 (B), which document the corresponding cytokine production of splenocytes which were restimulated with beta-Gal protein *in vitro*. BALB/c mice were immunized as already described above for fig. 2. The splenocytes were stimulated with beta-Gal protein *in vitro*, the corresponding culture supernatants were obtained and the IFN-gamma or IL-4 concentration was determined using an ELISA standard test.

Fig. 4 shows further graphical views which demonstrate the antibody response of mice immunized according to the invention. BALB/c mice were immunized as described for fig. 2. Two weeks after the boost, blood was taken and the blood serum was obtained therefrom. Beta-Gal-specific IgG1 (A) and IgG2a antibodies (B) were determined with the aid of an ELISA test. In each case the extinction (OD) at 405 nm which results from the conversion of the substrate ABTS in the ELISA test is shown on the y-axis. The extinctions shown are the values from

which the corresponding values of mice treated with injection buffer are subtracted.

Fig. 5 shows microscope sections, stained with X-Gal, of the auricula of mice which have been injected i.d. into the auricula with mRNA which codes for beta-galactosidase. 12 hours after the injection of 25 µg RNA in HEPES-NaCl injection buffer, the ears were removed and sections stained with X-Gal were prepared. Blue cells indicate a beta-galactosidase activity. As can be seen from the two sections, only few blue cells are present.

Fig. 6 shows a section, corresponding to fig. 5, through an auricula of a mouse which was injected into the auricula with mRNA which codes for beta-galactosidase and was stabilized with protamine. The microscope section stained with X-Gal show a few cells stained blue.

Fig. 7 shows two further sections through the auricula of mice, two images being produced per section in order to represent a larger area. In this case, mRNA which codes for beta-galactosidase, in a buffer, to which 10 U RNasin, an enzymatic RNase inhibitor from the pancreas (obtainable from Roche or Promega) was added directly before the injection, was injected into the auricula. Compared with the sections of fig. 5 and fig. 6, significantly more blue-stained regions of cells with beta-galactosidase activity are to be recognized.

Fig. 8 shows a schematical view of the plasmid pT7TS, which was used for the *in vitro* transcription. Constructs according to the invention were cloned into the BglII and SpeI sites, the relative position of which to one another is shown. The region shaded in black contains the 5' untranslated region of the beta-globin gene from *Xenopus laevis*, while the region shaded in grey represents a corresponding 3' untranslated region of the beta-globin gene from *X. laevis*. The relative position of the T7 promoter, the PstI site used for sequencing, the poly(A$^+$) tail ($A_{30}C_{30}$) and, with an arrow, the transcription direction are furthermore indicated.

Fig. 9 shows in a flow chart, by way of example, the course of an RNA vaccination therapy according to the invention with assisting administration of GM-CSF. The mRNA molecules which code for one or more tumour antigens (MUC1, Her-2/neu, tilomerase, MAGE-1) or a mRNA which codes for a control antigen (influenza matrix protein (IMP), a viral antigen) are administered i.d. to the patient on days 0, 14, 28 and 42. In addition, one day after the RNA inoculation the patient is injected s.c. with GM-CFS (Leucomax® (100 µg/m^2) from Novartis/Essex Pharma). When the course is stable or there is an objective tumour response (complete remission (CR) or partial remission (PR)), the patients receive the vaccinations s.c. once a month. After the fourth injection (day 49), the response of the

tumour is evaluated radiologically, by laboratory chemistry or sonographically, and the immunological phenomena induced by the therapy are evaluated. From day 70, the immunization therapy is continued at intervals of 4 weeks. On day 0, 14, 28, 42 and 49, blood samples are taken for determination of appropriate laboratory parameters, the differential blood count (Diff-BB), FACS analysis and cytokines. Restaging of the patient takes place from day 49 and where appropriate every further 4 to 8 weeks.

Fig. 10 shows a flow chart of the construction of autologous, stabilized RNA according to the preparation process of the present invention. Tumour tissue is first obtained, e.g. by biopsy. The total RNA is extracted from this. A cDNA library is constructed with the aid of the poly(A^+) RNA obtained from the RNA extraction. Starting from this, after preparation of a corresponding DNA matrix, the autologous, stabilized RNA is obtained by means of *in vitro* transcription.

Fig. 11 shows a reaction scheme of the steps for preparation of a cDNA library, starting from poly(A^+) RNA, for the SMART PCR cDNA Synthesis Kit from Clontech Inc. by way of example.

Fig. 12 shows a photograph of an agarose gel which shows the typical size fractionation of a cDNA library compiled from human placenta tissue. A length marker with fragments of the length shown on the left is plotted in track M. The "DS cDNA" track

contains the cDNA library. Those fragments which correspond to the expected size fraction (about 200 bp to 4,000 bp) are used for the *in vitro* transcription.

Fig. 13 shows by way of example a treatment plan for the tumour therapy according to the invention by injection of a tumour mRNA library, here in combination with GM-CSF, for patients with malignant melanoma. Autologous, stabilized RNA prepared from the patient's own tumour tissue is used for this. This amplified autologous tumour RNA is administered to the patient i.d. on days 0, 14, 28 and 42. In addition, one day after the RNA injection the patient is injected s.c. with GM-CSF (Leucomax® 100 µg/m^2 Novartis/Essex Pharma). Two weeks after the fourth injection (day 56), the response of the tumour is evaluated by a staging analysis (inter alia sonography, thorax X-ray, CT etc.) and by assessment of the immunological parameters induced by the therapy. When the course of the disease is stable or there is an objective tumour response (CR or PR), the patient receives in each case a further vaccination every four weeks. Further restaging analyses are carried out on day 126 and then at intervals of 12 weeks.

Fig. 14 shows once more schematically of the general course of a therapy with the pharmaceutical composition according to the invention with autologous, amplified tumour RNA, i.e. the RNA contained in the pharmaceutical composition represents a cDNA

library of the tumour tissue. A sample of the tumour tissue is first obtained, e.g. via a biopsy. The total and then the poly(A^+) RNA are prepared from the tissue by appropriate extractions. Starting from the poly(A^+) RNA, a cDNA library is constructed and is cloned into a vector suitable for subsequent *in vitro* transcription. An RNA vaccine is then obtained by *in vitro* transcription, and is injected into the patient from whom the tumour tissue has been taken to combat the tumour.

The following embodiment examples explain the present invention in more detail, without limiting it.

EXAMPLES

Example 1: Tumour vaccination with RNA in an animal model

Materials and methods

Capped mRNA which codes for a shortened version of the Her-2/neu protein of the rat ("ECD-TM-neu-rat", containing the extracellular domain and the transmembrane region, but not the cytoplasmic region) was prepared, using the "SP6 mMessagemMachine" (Ambion), with the aid of a plasmid which substantially corresponded to the structure shown in fig. 8, but contained an SP6 promoter instead of the T7 promoter and in which the ECD-TM-neu-rat construct was inserted after the SP6 RNA polymerase promoter. The mRNA prepared was dissolved in injection buffer (150 mM NaCl, 10 mM HEPES) at a concentration of 0.8 mg/l and the solution was mixed with protamine sulfate (Sigma) (1 mg

protamine per 1 mg RNA). 50 µl of this solution were injected into the auriculae (in each case 25 µl per ear) of mice. Eight injections were performed, in each case one at the age of 6, 8, 13, 15, 20, 22, 27 and 29 weeks. Mice to which corresponding injections with injection buffer, with plasmid DNA which codes for ECD-TM-neu rat or with an antisense mRNA corresponding to the mRNA according to the invention were administered served as controls.

Results

Female BalB-neu T mice (BalB/c mice which express the oncogene Her-2/neu of the rat; cf. Rovero et al. (2000) J. Immunol. 165(9):5133-5142) which develop mammary carcinomas spontaneously were immunized with RNA which codes for a shortened version of the Her-2/neu protein ("ECD-TM-neu-rat", containing the extracellular domains and the transmembrane region, but not the cytoplasmic region). Four mice treated with injection buffer served as a negative control. A further group of three mice was injected with DNA which codes for the shortened Her-2/neu. Four mice received the mRNA which codes, according to the invention, for the tumour antigen Her-2/neu (shortened version of ECD-TM, see above). Four mice which were injected with the corresponding antisense RNA served as a further control group. As shown in fig. 1, in the animals of the untreated control group a tumour multiplicity of on average 10 was observed after 26 weeks, whereby all animals having palpable breast tumours at the age of about 20 weeks. In contrast, in the case of immunization with the mRNA which codes for ECD-TM-neu-rat, a significant slowing down of the formation of carcinomas is to be observed, in particular a

tumour multiplicity of 10 is achieved only at the age of 30 weeks. Furthermore, the size of the tumours is also reduced (not shown). Of the 4 mice treated with the mRNA according to the invention, one was still tumour-free after 9 months. That group of mice which had been injected with the antisense mRNA all showed tumours at the age of 6 months. The comparison group of mice injected with plasmid DNA which codes for the shortened version of Her-2/neu also showed a carcinoma formation which was slowed down compared with the untreated control group (cf. also in respect of corresponding plasmid DNA experiments on intramuscular injection: Di Carlo et al. (2001) Clin. Cancer Res. 7 (3rd supplement): 830s-837s), but the formation of carcinomas up to the 27th week was not slowed down to the same extent as in the case of immunization with mRNA according to the invention which codes for the shortened version of Her-2/neu. Furthermore, in the case of immunization with DNA, the abovementioned disadvantages, in particular the risk of integration of the DNA into the genome, the formation of anti-DNA antibodies etc., are to be taken into account.

Example 2: Influence of GM-CSF on RNA vaccination

Materials and methods

Mice

BALB-c AnNCrlBR (H-2^d) mice (female) 6-10 weeks old were obtained from Charles River (Sulzfeld, Germany).

Plasmids and preparation of RNA

The ORF (LacZ) which codes for beta-galactosidase, flanked by 5'-and 3'-untranslated sequences from the beta-globin gene of *X. Laevis*, was into the plasmid pT7TS (P.A. Creek, Austin, TX, USA), in order to prepare the plasmid pT7TS-kozak-5' beta gl-lacZ-3' beta gl-A30C30 (cf. Hoerr et al. (2000) Eur. J. Immonol. 30: 1-7). A schematical view of the general structure of the plasmid pT7TS with the flanking 5' and 3' untranslated sequences from the beta-globin gene of *X. Laevis* is shown in fig. 8.

The plasmid prepared in this way was linearized with PstI and transcribed *in vitro* using the m-MessagemMachineT7 Kit (Ambion, Austin, TX USA). The RNA prepared in this way was purified by means of LiCl precipitation, phenol/chloroform extraction and ammonium acetate precipitation. Finally, the purified RNA was resuspended in injection buffer (150 mM NaCl, 10 mM HEPES) in a concentration of 0.5 mg/ml.

Media and cell culture

P815 and P13.1 cells were cultured in RPMI 1640 (Bio-Whittaker, Verviers, Belgium), supplemented with 10% heat-inactivated foetal calf serum (FCS) (PAN systems, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

CTL cultures were kept in RPMI 1640 medium, supplemented with 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.05 µM beta-mercaptoethanol, 50 mg/ml gentamycin, MEM non-essential amino acids (100 x)

and 1 mM sodium pyruvate. The CTL were restimulated for one week with 1 mg/ml beta-galactosidase protein (Sigma, Taufkirchen, Germany). On day 4, 4 ml of culture supernatant were carefully pipetted off and replaced by fresh medium containing 10 U/ml rIL-2 (final concentration).

Immunization

3 BALB/c mice per group were anesthetized with 20 mg pentobarbital i.p. per mouse. The mice were then injected i.d. in both auriculae with 25 mg of mRNA which codes for beta-galactosidase (beta-Gal) in injection buffer (150 mM NaCl, 10 mM HEPES). In some cases, granulocyte macrophage colony-stimulating factor (GM-CSF) was additionally injected into the same site or into an injection site away from this (into the auricula or s.c. into the back) 24 h or 2 h before or 24 h after the RNA injection. As a positive control, animals were injected i.d. in both auriculae with in each case 10 mg of a DNA plasmid which codes for beta-gal in PBS. A group of animals to which only injection buffer was administered i.d. into both auriculae served as a negative control. Two weeks after the first injection, a boost injection was performed in each case in the same manner as the first injection. Two weeks after the boost injection, blood was taken, the mice were sacrificed and the spleen was removed.

^{51}Cr release test

Splenocytes obtained from the spleen were stimulated with beta-gal protein *in vitro* and the CTL activity was

determined after 6 days using a 6-hours ^{51}Cr standard test as described in Rammensee et al. (1989) Immunogenetics 30: 296 - 302. Summarized briefly, target cells were marked with ^{51}Cr and charged with the peptide TPHPARIGL for 20 min at room temperature. After co-incubation of effector and target cells (at in each case three different ratios of effector:target cells: 200, 44 and 10) in circular plates with 96 wells for 6 h, 50 ml of 200 ml of culture supernatant were pipetted into a Luma scintillation plate (Packard) with 96 wells and, after drying, the radioactivity was measured with a scintillation counter (1405 Microbeta Plus). The percentage specific release was determined from the amount of ^{51}Cr released into the medium (A) minus the spontaneous release (B) divided by the total release (C) (using Triton X-100) minus the spontaneous release (B): Per cent specific lysis = 100 (A-B)/(C-B).

Cytokine ELISA

After 4 days of restimulation with beta-gal protein, the supernatant of the splenocyte culture was pipetted off and stored at -50 °C until used. 100 ml anti-mouse-anti-IFN-gamma or -IL-4 scavenger antibodies (Becton Dickenson, Heidelberg, Germany) were pipetted out overnight at 4 °C on MaxiSorb plates (Nalge Nunc International, Nalge, Denmark) at a concentration of 1 mg/ml in coating buffer (0.02 % NaN_3, 15 mM Na_2CO_3, 15 mM $NaHCO_3$, pH 9.6). After washing three times with washing buffer (0.05 % Tween 20 in PBS), the plates were saturated with 200 ml of blocking buffer (0.05 % Tween 20, 1 % BSA in PBS) for 2 h at 37 °C. After washing three times with washing buffer, 100 ml of the cell culture supernatants were incubated for 5 h at 37 °C. The

plates were then washed four times with washing buffer, 100 ml of biotinylated anti-mouse-anti-IFN-gamma or -IL-4 detection antibodies (Becton Dickenson, Heidelberg, Germany) per well at a concentration of 0.5 mg/ml in blocking buffer were pipetted and incubation was carried out for 1 h at room temperature. After washing three times with washing buffer, 100 ml of a 1/1,000 dilution of streptavidin-HRP (BD Biosciences, Heidelberg, Germany) were added into each well. After 30 min at room temperature, the plates were washed three times with washing buffer and twice with bidistilled water. Thereafter, 100 ml of the ABTS substrate were added into each well. After 30 min at room temperature, the extinction at 405 nm was measured with a Sunrise ELISA reader (Tecan, Crailsheim, Germany).

Antibody ELISA

Two weeks after the boost injection, blood was taken from the mice via the orbital vein and blood serum was prepared. 100 ml of beta-gal protein at a concentration of 100 mg/ml in coating buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.5) were pipetted out for 2 h at 37 °C on to MaxiSorb plates (Nalge Nunc International, Nalge, Denmark). The plates were then washed three times with 200 ml of washing buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.01 M EDTA, 0.1 % Tween 20, 1 % BSA, pH 7.4) and saturated with protein with 200 ml of washing buffer overnight at 4 °C. The plates were washed three times with washing buffer and blood sera were added in a dilution of 1/10, 1/30 or 1/90 in washing buffer. After 1 h at 37 °C, the plates were washed three times with washing buffer and 100 ml of 1/1,000 dilutions

of goat anti-mouse IgG1 or IgG2a antibodies (Caltag, Burlington, CA, USA) were added. After 1 h at room temperature, the wells were washed three times with washing buffer and 100 ml of ABTS substrate per well were added. After 15 - 30 min at room temperature, the extinction at 405 nm was measured with a Sunrise ELISA reader (Tecan, Crailsheim, Germany).

Results and discussion

It was confirmed that direct injection of RNA which codes for beta-galactosidase into the auricula of mice induces an anti-beta-galactosidase immune response, substantially of the Th2 type. Production of anti-beta-galactosidase immunoglobulins of the IG1 type (fig. 3A) and secretion of IL-4 (fig. 3B) was found in splenocytes, stimulated with beta-galactosidase, from mice which had been injected with the RNA which codes for beta-galactosidase. To increase the efficiency of the RNA vaccine, the cytokine GM-CSF was additionally administered. This cytokine increases the efficiency of some DNA vaccines. It was furthermore found that the time of the GM-CSF injection influences the type of the immune response, compared with DNA injection (Kusakabe (2000) J. Immunol. 164: 3102-3111). It was found according to the invention that GM-CSF can enhance the immune response brought about by an RNA vaccination. The injection of GM-CSF one day before the injection of RNA shows scarcely any influence on the strength or the type of the immune response. In contrast, injection of GM-CSF 2 hours before injection of the RNA enhances the immune response (cf. the IL-4 release in fig. 3B in the 2 mice injected with GM-CSF at time T = 0), but does not influence

the Th2 polarity. On the other hand, if GM-CSF is injected one day after the RNA vaccine into the same site or into a site away from this (not shown), not only is the immune response enhanced overall (cf. the antibody response according to fig. 3), the immune response is polarized to the Th1 type (cf. the IFN-gamma production by splenocytes stimulated with beta-gal protein according to fig. 3A, the production of IgG2a antibodies against beta-Gal according to fig. 3B and the production of activated CTL according to fig. 1). The injection of GM-CSF some minutes or some hours after the RNA injection should result in the same effect (enhancement and polarization) on the immune response.

Example 3: Effect of an RNase inhibitor on mRNA expression *in vivo*

Naked or protamine-associated or -complexed mRNA which codes for beta-galactosidase (prepared as described in example 2) was injected into the auricula of mice in an amount of 25 mg of RNA in injection buffer (150 mM NaCl, 10 mM HEPES). Further mice were injected with the mRNA which codes for beta-galactosidase, together with 10 U of the RNase inhibitor RNasin (an enzymatic RNase inhibitor extracted from the pancrease, obtainable from Roche or Promega). The RNase inhibitor was mixed with the RNA solution directly before the injection. After 12 hours, the ears were in each case removed from the mice. Thin microscope sections of the auriculae were prepared and were stained with X-gal. Injection of naked or protamine-associated mRNA leads to a detectable beta-galactosidase activity in a few cells in the corresponding thin sections (blue cells in fig. 5 and 6). Some cells have thus taken up

the exogenous RNA here and translated it into the protein. When the mRNA which codes for beta-galactosidase was in the form protected with the RNase inhibitor RNasin, very many more blue cells were observed than in the case of the naked or protamine-associated RNA (fig. 7). Since RNasin inhibits RNases, the half-life of the injected mRNA molecules *in vivo* is prolonged, where the environment (interstitial tissue) is contaminated with RNases. Such a stabilization of the RNA leads to an increased uptake by the surrounding cells and therefore to an increased expression of the protein coded by the exogenous RNA. This phenomenon can therefore also be utilized for an enhanced immune response to an antigen coded by the mRNA injected.

Example 4: RNA vaccination of patients with malignant diseases

Introduction

Cytotoxic T lymphocytes (CTL) recognize antigens as short peptides (8-9 amino acids) which are expressed bound to MHC class 1 glycoproteins on the cell surface (1). These peptides are fragments of intracellular protein molecules. However, there are indications that antigens taken up exogenously by macropinocytosis or phagocytosis can lead to the $CD8^+$ T cell-mediated immune response. The proteins are cleaved into proteosomes and the peptides formed by this means are transported out of the cytosol into the lumen of the endoplasmic reticulum and bound to MHC class I molecules.

The proteins processed in this way are transported as peptide/MHC class I complex to the cell surface and presented to the CTL. This process takes place in every cell and in this way makes it possible for the immune system to monitor accurately each individual cell for the presence of proteins which are foreign to the body or modified or embryonic, regardless of whether they originate from intracellular pathogenic germs, oncogenes or dysregulated genes. By this means, cytotoxic lymphocytes are capable of recognizing and lysing infected and neoplastic cells, respectively (2, 3).

In recent years various tumour-associated antigens (TAA) and peptides which are recognized by CTL and therefore lead to lysis of tumour cells have been successfully isolated (21-27). These TAA are capable of stimulating T cells and inducing antigen-specific CTL, if they are expressed as a complex of HLA molecule and peptide on antigen-presenting cells (APC).

In numerous studies carried out mainly on patients with malignant melanoma, it has been possible to demonstrate that malignant cells lose the expression of TAA as the tumour disease proceeds. Similar circumstances are also observed with vaccinations with individual tumour antigens. Under vaccination therapies, selection of tumour cells may also occur, which renders possible an escape from the immune system and a progression of the disease in spite of therapy. The use of several different tumour antigens as envisaged in the treatment plan according to the invention of the present example should prevent selection of tumour

cells and escape of the malignant cells from the immune system due to loss of antigens.

A method with which DC can be transfected with RNA from a plasmid which codes for a tumour antigen has recently been developed (Nair et al., 1998, Nair et al., 2000). Transfection of DC with RNA for CEA or telomerase led to induction of antigen-specific CTL. This process renders it possible to induce CTL and T helper cells against several epitopes on various HLA molecules from a tumour antigen. A further advantage of this strategy is the fact that neither the characterization of the tumour antigens or epitopes used nor definition of the HLA haplotype of the patient is a prerequisite. By a polyvalent vaccine of this type, the probability of the occurrence of so-called clonal "tumour escape" phenomena could be reduced significantly. Furthermore, T cell-mediated immune responses against antigens processed and presented by the natural route and with possibly a higher immune dominance could be induced by this approach. By additional participation of MHC class II-restricted epitopes, the induced tumour-specific immune response could be enhanced and maintained for longer.

A treatment sheme according to the invention for tumour vaccination of patients with advanced malignant diseases (mammary, ovarian, colorectal, pancreatic and renal cell carcinomas) is provided by way of example. In this, RNA which has been prepared from plasmids which code for MUC1, Her-2/neu, telomerase and MAGE-1 tumour antigens and influenza matrix protein (IMP) (positive control) is administered intradermally to patients with the abovementioned malignant diseases. A CTL induction *in vivo*

is thereby rendered possible, in order to prevent the progression of the disease or to effect the regression thereof in this way. The tumour antigens mentioned are expressed on the malignant cells of mammary, ovarian, colorectal, pancreatic and renal cell carcinomas.

According to the treatment plan (cf. the following statements in this respect and fig. 9), the RNA species prepared in the laboratory which code for CEA, MUC1, Her-2/neu, telomerase, Mage-1 and IMP are administered to the patient i.d., initially 4 x on days 0, 14, 28 and 42. In addition, GM-CSF (Leucomax®, 100 µg/m^2, Novartis/Essex Pharma) is administered s.c. to the patient in each case one day after the RNA inoculation.

The treatment according to the invention is an immunisation approach which requires only minimal interventions on the patient (injection). Therapy is conducted ambulant and is suitable for many tumour patients, without the limitation to particular HLA types or defined T cell epitopes. Furthermore, polyclonal CD4$^+$-T helpers and also CD8$^+$-CTL can be induced by this therapy.

Treatment plan

The RNAs for several tumour antigens (MUC1, Her-2/neu, telomerase, MAGE-1) and for a control antigen, influenza matrix protein (IMP, a viral antigen) are administered i.d. to the patient on days 0, 14, 28 and 42. In addition, the patients receive GM-CSF (Leucomax® (100 µg/m^2) Novartis/Essex Pharma) s.c. in each case one day after the RNA inoculation. When the course of the disease is stable

or there is an objective tumour response (complete remission (CR) or partial remission (PR)), where appropriate the patients receive the vaccinations s.c. once a month. After the fourth injection (day 49), the response of the tumour is evaluated radiologically, by laboratory chemistry and/or sonographically, and the immunological phenomena induced by the therapy are evaluated.

From day 70, the immunization therapy is continued at intervals of 4 weeks.

On days 0, 14, 28, 42 and 49, in each case blood samples are taken for laboratory parameters, Diff-BB, FACS analysis and cytokines (50 ml in total). Restaging of the patients takes place from day 49 and where appropriate every further 4 to 8 weeks.

The treatment plan is shown schematically in fig. 9.

Laboratory: clotting, electrolytes, LDH, β2-M, CK, liver enzymes, bilirubin, creatinine, uric acid, total protein, CRP, tumour markers (Ca 12-5, Ca 15-3, CEA, Ca 19-9): 15 ml of blood.

Diff-BB: differential blood count with smear (5 ml of EDTA blood).

Cytokines: 10 ml of serum

FACS: 10 ml of heparin blood.

ELIspot: 20 ml of heparin blood.

Multitest: analysis of the DTH reaction.

DTH: ("delayed type hypersensitivity", delayed T cell-mediated reaction) analysis of the reaction to intradermally administered RNA. In addition a skin

biopsy should be performed in the event of a positive DTH reaction (local anaesthesia is not necessary for this).

Preparation of RNA from plasmids

For production of a vaccine based on mRNA, only precursors which are chemically synthesized and purified from bacteria are required. This is preferably effected in a specially equipped RNA production unit. This is in a sealed-off room which is declared an RNase-free zone, i.e. work with RNase (e.g. purification of plasmids) must not be carried out. Contamination with naturally occurring RNases is also constantly checked. This room is fitted out with new apparatuses (4 °C and -20 °C refrigerators, heating block, sterile bench, centrifuges, pipettes) which have never been used for biological or clinical work. This RNA production unit is used exclusively for enzymatic production (*in vitro* transcription) of mRNA (without bacterial, viral or cell culture work). The end product comprises a sterile RNA solution in HEPES/NaCl buffer. Quality analyses are carried out on a formaldehyde-agarose gel. In addition, the RNA concentration and the content of proteins are determined photometrically (OD_{320} < 0.1; ratio of OD_{260}/OD_{280} > 1.8 in pure RNA). Possible contamination by LPS is analysed in the LAL test. All RNA samples are subjected to sterile filtration before administration.

Plasmid constructs

The chosen genes (CEA, mucin1, Her-2/neu, telomerase, Mage-A1 and influenza matrix) are amplified via a PCR using

a heat-stable high-performance enzyme (pfu, Stratagene). The genes originate from tumour cDNA (mucin1, Her-2/neu, telomerase), or they have been cloned into bacterial vectors (influenza matrix and MAGE-A1). The PCR fragments are cleaved with restriction enzymes (mucin1: BglII-SpeI; Her-2/neu: HinDIIIblunt-SpeI; telomerase: BglII-SpeI; MAGE-A1: BamHI-SpeI; influenza matrix protein: BglII-SpeI) and cloned into the T7TS-Plasmid (cf. fig. 8) via the BglII and SpeI restriction sites. Plasmids of high purity are obtained via the Endo-free Maxipreparation Kit (Qiagen, Hilden, Germany). The sequence of the vector is controlled via a double-strand sequencing from the T7 promoter up to the PstI site and documented. Plasmids with a correct inserted gene sequence without mutations are used for the *in vitro* transcription. (Control via the published sequences: Accession Numbers: M11730 for Her-2/neu, NM_002456 for MUC1, NM_003219 for telomerase TERT, V01099 for influenza matrix and M77481 for MAGE-A1).

in vitro Transcription

Production of linear, protein-free DNA

500 µg of each plasmid are linearized in a volume of 0.8 ml via digestion with the restriction enzyme PstI in a 2 ml Eppendorf reaction vessel. This cleaved construct is transferred into the RNA production unit. 1 ml of a mixture of phenol/chloroform/isoamyl alcohol is added to the linearized DNA. The reaction vessel is vortexed for 2 minutes and centrifuged at 15,000 rpm for 3 minutes. The aqueous phase is removed and mixed with 0.7 ml 2-propanol in a 2 ml reaction vessel. This vessel is centrifuged at

15,000 rpm for 15 minutes, the supernatant is discarded and 1 ml 75% ethanol is added. The reaction vessel is centrifuged at 15,000 rpm for 10 minutes and the ethanol is removed. The vessel is centrifuged for a further 2 minutes and the residues of the ethanol are removed with a microlitre pipette tip. The DNA pellet is then dissolved in 1 µg/ml in RNase-free water.

Enzymatic synthesis of the RNA

The following reaction mixture is prepared in a 50 ml Falcon tube: 100 µg linearized protein-free DNA, 1 ml 5x buffer (200 mM Tris-HCl (pH 7.9), 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, 50 mM DTT), 200 µl ribonuclease (RNase) inhibitor (recombinant, 5,000 U), 1 ml rNTP mix (in each case 10 mM ATP, CTP, UTP; 2 mM GTP), 1 ml CAP analogue (8 mM), 150 µl T7 polymerase (3,000 U) and 2.55 ml RNase-free water. The total volume is 5 ml. The mixture is incubated at 37 °C for 2 hours in a heating block. Thereafter, 100 U of RNase-free DNase are added and the mixture is incubated again at 37 °C for 30 minutes. The DNA matrix is enzymatically degraded by this procedure.

Description and origin of the individual components

T7 polymerase: purified from an *E .coli* strain which contains a plasmid with the gene for the polymerase. This RNA polymerase uses as the substrate only promoter sequences of the T7 phage; Fermentas.

NTPs: synthesized chemically and purified via HPLC. Purity more than 96 %; Fermentas.

CAP analogue: synthesized chemically and purified via HPLC. Purity more than 90 %; Institute of Organic Chemistry of the University of Tübingen.
RNase inhibitor: RNasin, for injection, prepared recombinantly (*E. coli*); Promega.
DNase: Pulmozym® ("dornase alfa"); Roche

Purification

The RNA treated with DNase is mixed with 20 ml of a solution of 3.3 ml 5 M NH_4OAc plus 16.65 ml of ethanol. The mixture is incubated at -20 °C for 1 hour and centrifuged at 4,000 rpm for 1 hour. The supernatant is removed and the pellet is washed with 5 ml of 75 % RNase-free ethanol. The vessel is centrifuged again at 4,000 rpm for 15 minutes and the supernatant is removed. The vessel is centrifuged again under the previous conditions and the ethanol which remains is removed with a microlitre pipette tip. The reaction vessel is opened and the pellet is dried under a sterile bench in the sterile environment.

1 ml of RNase-free water is added to the dried RNA. The pellet is incubated at 4 °C for at least 4 hours. 2 µl of the aqueous solution are subjected to a quantitative analysis (determination of the UV absorption at 260 nm). 2 ml of a phenol/chloroform/isoamyl alcohol solution are added to 1 ml of aqueous RNA solution. The mixture is vortexed for 2 minutes and centrifuged at 4,000 rpm for 2 minutes. The aqueous phase is removed with a microlitre pipette and transferred into a new reaction vessel. 4 ml of a solution of 0.66 ml 5 M NH_4OAc plus 3.33 ml ethanol are added. The mixture is incubated at -20 °C for 1 hour and

centrifuged at 4,000 rpm for 1 hour. The supernatant is removed and the pellet is washed with 75 % RNase-free ethanol. The vessel is centrifuged again at 4,000 rpm for 15 minutes and the supernatant is removed. The vessel is centrifuged again under the previous conditions and the ethanol which remains is removed with a microlitre pipette tip. The reaction vessel is opened and the pellet is dried under a sterile bench in the sterile environment.

The RNA is dissolved in RNase-free water and adjusted to a concentration of 10 mg/ml. It is incubated for 12 hours at 4 °C. A final concentration of 2 mg/ml is achieved by addition of injection buffer (150 mM NaCl, 10 mN HEPES). The end product is preferably subjected to sterile filtration under GMP conditions before use.

Application of the RNA

Each patient receives at two different sites an intradermal (i.d.) injection of in each case 150 µl of the injection solution in which in each case 100 µg of antigen-coding mRNA (CEA, Her-2/neu, MAGE-A1, mucin 1, telomerase, influenza matrix protein) are present in solution.

After the primary immunization, a booster immunization is carried out every 14 days, for the inoculations then to be repeated at a monthly interval. In each case one day after the RNA injection, GM-CSF (Leucomax®, Sandoz/Essex Pharma) is administered subcutaneously (s.c.) to the patient.

If a clinical response is present or the disease is stabilized, this therapy is continued at monthly intervals.

Further immunological investigations *in vitro* (optional)

Flow cytometry analyses of PBMC for quantification of CTL precursors;
^{51}Cr release tests;
Soluble receptor and cytokine levels in the serum;
DTH reaction (skin reaction to intradermally injected RNA, "delayed type hypersensitivity", T lymphocyte-mediated reaction); and
Skin biopsy samples from the injection site for histological analysis for T cell infiltration (pathology).

Parameters for evaluation of the efficacy

To be able to answer the question of the efficacy of this immunotherapy, the induction of tumour-specific T cells and a measurable tumour remission is used. Parameters are T cell reactions measured *in vitro* and *in vivo* and changes in the size of bidimensionally recordable tumour manifestation or laboratory chemistry parameters of the course of the disease.

Objective remission is defined as the best response in the form of a complete or partial remission, corresponding to the criteria listed below. The remission rate is calculated from the ratio of the number of patients with objective remission and the total number of evaluable patients.

A change in the immune status, determined by immunotyping of peripheral mononuclear cells, an increase in the antigen-specific CTL precursor frequency in the peripheral

blood and the induction of a persistent tumour-specific T cell activity are assessed as the immunological response to the therapy. For this purpose, *in vitro* induction cultures are established for activation of tumour-specific CTL.

Remission criteria (acc. to UICC)

Complete remission (CR):	Complete regression of all measurable tumour manifestation, documented by 2 control investigations at least 4 weeks apart.
Partial remission (PR):	Decrease in size of the total area dimensions (product of two tumour diameters or linear measurement of one-dimensionally measurable lesions of all tumour findings by 50 % for at least 4 weeks). No new occurrence of tumour manifestations or progression of a tumour finding.
"No Change" (NC):	Decrease of all the measurable tumour manifestations by less than 50 % or increase in a tumour finding.
Progression (PD):	Increase in size of the tumour parameters in at least one focus or new occurrence of a tumour manifestation.

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Example 5: Vaccination with autologous, amplified tumour RNA in patients with malignant melanoma

Introduction

The incidence of malignant melanoma has increased sharply worldwide in recent years. If the melanoma disease is already in the metastased stage at the time of diagnosis, there is currently no therapy which has a positive influence on the further course of the disease with sufficient certainty.

Vaccination therapies carried out to date using dendritic cells are very labour-, cost- and time-intensive because of the complicated culturing of the cells (GMP conditions). Furthermore, the studies have hitherto concentrated predominantly on known tumour-associated antigens (TAA), such as, for example, melan-A or tyrosinase.

A number of various immunological phenomena, such as, inter alia, the occurrence of spontaneous tumour regressions or spontaneous involution of metastases, have made the melanoma the prior candidate for testing immunotherapy investigations (Parkinson et al., 1992). In addition to experiments on non-specific stimulation of the immune system by means of interleukin-2, mistletoe extracts, BCG and interferons, which have so far not led to decisive breakthroughs in the therapy of advanced tumour diseases, the strategy of induction of various highly specific cytotoxic T lymphocytes (CTL) has been pursued in particular in recent years. These CTL are capable of recognizing and killing autologous melanoma cells (Boon et al., 1994; Houghton, 1994). Studies of this process have shown that the CTL recognize defined peptides in combination with MHC class I molecules. The presentation of peptides by antigen-presenting cells (APC) is the physiological route to generation of specific immune responses by lymphocytes (Rammensee, 1993). Dendritic cells have proved to be potent antigen-presenting cells which lead to an induction of the immune response by two routes: The first is the direct presentation of peptides towards $CD8^+$-T lymphocytes and activation thereof (Schuler & Steinmann, 1985; Inaba et al., 1987; Romani et al., 1989), and the second is the generation of a protective immune response, which is mediated by $CD4^+$ helper lymphocytes, and requires a presentation of peptides via MHC class II molecules (Grabbe et al., 1991, 1992, 1995).

By means of peptide analysis, it was therefore possible to identify in this way various tumour-associated antigens (TAA) which are specific for the melanoma and, after

presentation in combination with the MHC molecule and recognition by the CTL, lead to cytolysis of the tumour cells (Schadendorf et al., 1997, p. 21-27).

The use of autologous, dendritic cells was tested in the context of a pilot study on melanoma patients in respect of its potential to induce cytotoxic T lymphocytes effectively, rapidly and reliably. In this study, 16 melanoma patients in stage IV who had already been pretreated by chemotherapy were vaccinated with peptide-charged dendritic cells. The response rates were above 30 % (5/16 patients) (Nestle et al., 1998). In a further independent study it was possible to demonstrate an even higher response rate of more than 50 % (6/11 patients) after immunization of melanoma patients who had already been pretreated by chemotherapy with MAGE-3A1-charged dendritic cells (Thurner et al., 1999). A significant expansion of MAGE-A3-specific $CD8^+$-T cells was also observed in 8/11 patients. A regression of the metastases took place in some cases after the DC vaccination. This was accompanied by a $CD8^+$-T cell infiltration. This showed that the T cells induced were active *in vivo*. A disadvantage of this strategy is the high outlay on costs and the laboratory (in particular GMP conditions). Large amounts of blood from the patient are required for the time-intensive generation of the DC. In the preparation of the peptides, on the one hand only known tumour-associated antigens can be used, and on the other hand various peptides are necessary, depending on the HLA haplotype.

A further development of this approach is vaccination with RNA-transfected DC (Nair et al., 1998, Nair et al., 2000).

In the meantime, numerous studies demonstrate that DC from mice and humans which have been transfected with mRNA can induce an efficient CTL response *in vitro* and *in vivo* and can lead to a significant reduction in metastases (Boczkowski et al., 1996, 2000; Ashley et al., 1997; Nair et al., 1998, 2000; Heiser et al., 2001; Mitchell and Nair, 2000; Koido et al., 2000; Schmitt et al., 2001). A great advantage in the use of RNA compared with peptides is that the most diverse peptides can be processed and presented from one mRNA which codes for a TAA. By a polyvalent vaccine of this type, the probability of the occurrence of so-called clonal "tumour escape" phenomena can be reduced significantly. Furthermore, T cell-mediated immune responses against antigens processed and presented by the natural route and with potentially a higher immune dominance can be induced by this system. By additional participation of MHC class II-restricted epitopes, the tumour-specific immune response induced can be intensified and maintained for longer. Nevertheless, this process also can be carried out only with a high outlay on the laboratory (GMP conditions) because of the necessary culturing of the autologous DCs.

In the present strategy according to the invention, vaccination is carried out with the RNA expression profile present in the autologous tumour of the patient. The specific tumour profile of the patient is thereby taken into account, unknown TAAs also being included in the inoculation. Expensive culture of the DCs is omitted, since RNAs (not transfected DCs) are used in the vaccination.

A vaccination therapy using amplified autologous tumour RNA on patients with metastased malignant melanoma, in particular stage III/IV, is therefore provided according to the invention.

Tumour-specific cytotoxic T cells are induced *in vivo* by the vaccination, in order thus to achieve a clinico-therapeutic effect (tumour response). This is an immunisation system which requires only minimal interventions on the patient (injection). Therapy can be conducted ambulant and is suitable for many tumour patients, without the limitation to particular HLA types or defined T cell epitopes. Furthermore, polyclonal $CD4^+$-T helpers and also $CD8^+$-CTL can be induced by this therapy. From the point of view of the strategy, it is decisive also that hitherto unknown TAAs are taken into account in the vaccination protocol, and the exclusive use of autologous material is particularly advantageous.

Treatment plan

The amplified autologous tumour RNA is administered to the patient i.d. on days 0, 14, 28 and 42. In addition, the patients receive GM-CSF (Leucomax® 100 µg/m^2, Novartis/Essex) s.c. in each case one day after the RNA inoculation. Each patient receives, at two different sites, an i.d. injection of in each case 150 µl of the injection solution, in which in each case 100 µg of autologous tumour RNA is dissolved.

2 weeks after the fourth injection (day 56), where appropriate the response of the tumour is evaluated by a

staging analysis (inter alia sonography, thorax X-ray, CT etc.; in this context see the statements below) and by assessment of the immunological parameters induced by the therapy.

When the course of the disease is stable or there is an objective tumour response (CR or PR), the patients receive the vaccinations every four weeks. Further restaging analyses can be envisaged e.g. on day 126 and then at an interval of 12 weeks.

A diagram of the treatment plan is shown in fig. 13.

Preparation of autologous tumour RNA

The aim is the preparation of autologous poly(A$^+$) RNA. For this, poly(A$^+$) RNA is isolated from the patient's own tumour tissue. This RNA isolated is very unstable per se and its amount is limited. The genetic information is therefore transcribed into a considerably more stable cDNA library and thus conserved. Starting from the patient's own cDNA library, stabilized autologous RNA can be prepared for the entire treatment period. The procedure according to the invention is shown schematically in fig. 10.

Isolation of RNA

A process of Roche AG is used to isolate total RNA from a tumour tissue biopsy. The High Pure RNA Isolation Kit (order number 1828665) is employed here in accordance with the manufacturer's instructions. Poly(A$^+$) RNA is isolated

from the total RNA via a further process of Roche AG with the High Pure RNA Tissue Kit (order number 2033674).

Preparation of a cDNA library

The cDNA library is constructed with the "SMART PCR cDNA Synthesis Kit" (Clontech Inc., USA; order number PT3041-1) in accordance with the manufacturer's instructions.

In this procedure, the single-stranded poly(A^+) RNA is subjected to reverse transcription via a specific primer. Via a poly-C overhang at the 3'-end of the newly synthesized DNA, a further primer can hybridize, via which the construct can be amplified by a PCR. The double-stranded cDNA fragments are now ready for cloning into a suitable RNA production vector (e.g. pT7TS; cf. fig. 8).

The process for the preparation of the cDNA library from the poly(A^+) RNA with the aid of the above kit is shown schematically in fig. 11.

Plasmid constructs

The cDNA PCR fragments are cleaved with the restriction enzymes NotI and SpeI and cloned into the corresponding restriction sites of the pT7TS vector by a procedure analogous to that described in example 4. Plasmids of high purity are obtained via the Endo-free Maxipreparation Kit (Qiagen, Hilden, Germany). Plasmids with a cloned-in gene sequence which corresponds to the expected size fractionation (200 bp - 4,000 bp) of the cDNA library are used for the *in vitro* transcription. An example of a

separation of a representative cDNA library in an agarose gel is shown in fig. 12.

in vitro Transcription and RNA administration

The *in vitro* transcription and the administration of the RNA are carried out as described in the above example 4.

Investigations during the treatment

Before each inoculation (on the day of the inoculation):
Physical examination (including RR, fever);
Blood sample for routine laboratory values
1. Blood count, differential blood count: 3 ml
2. Electrolytes, LDH, CK, liver enzymes, bilirubin, creatinine, uric acid, total protein, CRP: 5 ml
3. Blood sedimentation: 2 ml; and

at repeat inoculations additionally: Inspection of the injection sites.

On day 1 after each inoculation:
Physical examination (including RR, fever); and
Inspection of the injection sites.

In staging analyses on day 56 and 126 after the first inoculation, then every 12 weeks, additionally:
Extended routine blood sample:
1. Tumour marker S100 (7 ml)
2. Clotting values (3 ml);

Blood sample for immune monitoring (30 ml);
General well-being (ECOG score);

Imaging methods (thorax X-ray, sonography, skeleton scintigram, CT abdomen, pelvis, thorax, skull); and ECG ("EKG").

Further immunological investigations *in vitro*

Where appropriate, the relative incidence of antigen-specific CTL precursor cells in the peripheral blood of the patient in the course of time of the vaccination therapy is measured.

On the one hand CTL precursor cells which are directed against antigens expressed to a particular degree by melanoma cells (tyrosinase, MAGE-3, melan-A, GP100) are quantified here with FACS analyses (tetramer staining). On the other hand ELIspot analyses are carried out, these being designed such that CTL precursor cells which are directed specifically against hitherto unknown antigens are additionally recorded. For this, autologous dendritic cells cultured from the peripheral blood of the patient are incubated with the same RNA with which the inoculation has also been carried out. These then serve as stimulator cells in the ELIspot analysis. The measurement thus records the total vaccine spectrum. For these analyses, blood samples of 30 ml in total (20 ml ELIspot, 10 ml FACS analysis) can be envisaged for the immune monitoring in the context of the staging analyses and additionally on days 0, 14, 28 and 42, as well as a single withdrawal of 100 ml on day 70 for culture of the DC.

Furthermore, skin biopsy samples from the injection site can be obtained for histological analysis in respect of a T cell infiltration.

Parameters for evaluation of the efficacy

The efficacy of the therapy according to the invention is evaluated with the aid of the parameters described above in example 4.

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Amended claims

1. Pharmaceutical composition comprising at least one mRNA comprising at least one coding region for at least one antigen from a tumour, wherein the mRNA of at least one contains at least one analogue of nucleotides which actually occur, in combination with an aqueous solvent.

2. Pharmaceutical composition according to claim 1, wherein the coding region for the antigen(s) from a tumour and/or the 5' and/or the 3' untranslated region of the mRNA is modified compared with the wild-type mRNA, wherein the modification(s) stabilize(s) the mRNA.

3. Pharmaceutical composition according to claim 2, wherein the modification(s) has/have an effect on the translation efficiency of the mRNA.

4. Pharmaceutical composition according to one of claims 1 to 3, wherein the analogue is chosen from the group consisting of phosphorothioates, phosphoroamidates, peptide nucleotides, methylphosphonates, 7-deazaguanosine, 5-methylcytosine and inosine.

5. Pharmaceutical composition according to one of claims 1 to 4, wherein the mRNA has a 5'-cap structure and/or a poly(A^+) tail of at least about 25 nucleotides and/or at least one IRES and/or at least one 5'-stabilizing sequence and/or at least one 3'-stabilizing sequence.

6. Pharmaceutical composition according to claim 5, wherein the 5'- and/or the 3'-stabilizing sequence(s) is/are chosen from the group consisting of untranslated sequences (UTR) of the β-globin gene and a stabilizing sequence of the general formula $(C/U)CCAN_xCCC(U/A)Py_xUC(C/U)CC$.

7. Pharmaceutical composition according to one of claims 1 to 6, wherein the antigen(s) from a tumour is/are a polyepitope of antigens from a tumour.

17. Pharmaceutical composition according to one of claims 1 to 16, which comprises a plurality of mRNA molecules which represent a cDNA library, or a part thereof, of a tumour tissue.

18. Pharmaceutical composition according to claim 17, wherein the part of the cDNA library codes for the tumour-specific antigens.

19. Pharmaceutical composition according to one of claims 1 to 18, wherein the antigen(s) from a tumour is/are chosen from the group consisting of 707-AP, AFP, ART-4, BAGE, β-catenin/m, Bcr-abl, CAMEL, CAP-1, CASP-8, CDC27/m, CDK4/m, CEA, CT, Cyp-B, DAM, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, Gp100, HAGE, HER-2/neu, HLA-A*0201-R170I, HPV-E7, HSP70-2M, HAST-2, hTERT (or hTRT), iCE, KIAA0205, LAGE, LDLR/FUT, MAGE, MART-1/melan-A, MC1R, myosin/m, MUC1, MUM-1, -2, -3, NA88-A, NY-ESO-1, p190 minor bcr-abl, Pml/RARα, PRAME, PSA, PSM, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, TEL/AML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2 and WT1.

20. Pharmaceutical composition according to one of claims 1 to 19, comprising at least one further pharmaceutically acceptable carrier and/or at least one further pharmaceutically acceptable vehicle.

21. Pharmaceutical composition according to one of claims 1 to 20, for therapy and/or prophylaxis of cancer.

22. Process for the preparation of a pharmaceutical composition according to one of claims 1 to 21, comprising the steps:

(a) preparation of a cDNA library, or a part thereof, from tumour tissue of a patient,

(b) preparation of a matrix for *in vitro* transcription of RNA with the aid of the cDNA library or a part thereof and

(c) *in vitro* transcribing of the matrix.

23. Process according to claim 22, wherein the part of the cDNA library of the tumour tissue codes for the tumour-specific antigens.

24. Process according to claim 23, in which the sequences of the tumour-specific antigens are determined before step (a).

25. Process according to claim 24, wherein the determination of the sequences of the tumour-specific antigens comprises an alignment with a cDNA library from healthy tissue.

26. Process according to claim 24 or 25, wherein the determination of the sequences of the tumour-specific antigens comprises a diagnosis by a microarray.

Application number / numéro de demande: EP02/14577

Figures: 5, 6, 7, 12

Pages:

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)

Fig. 1

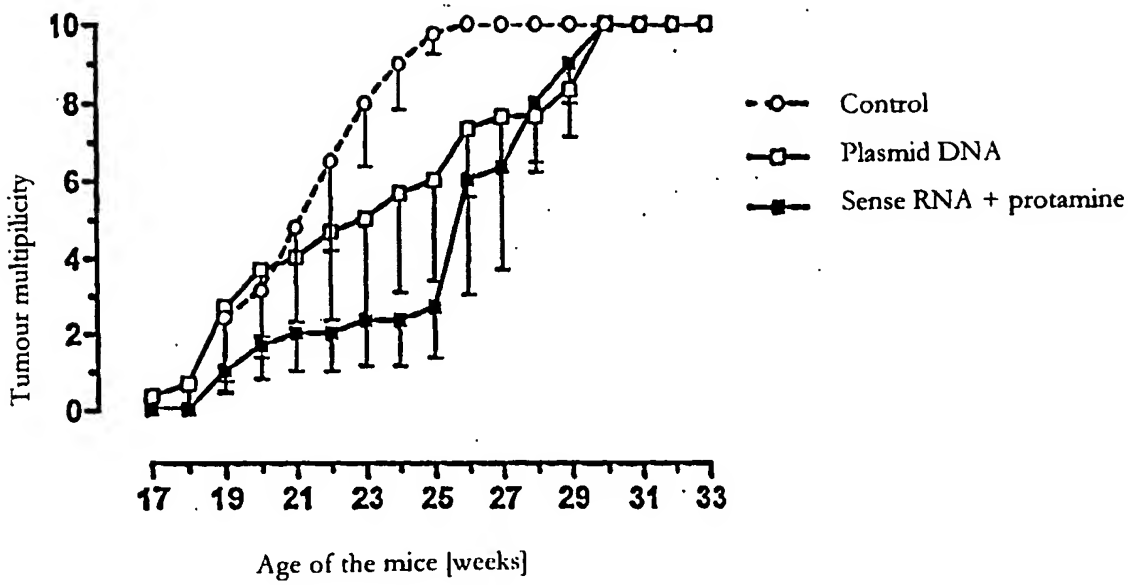
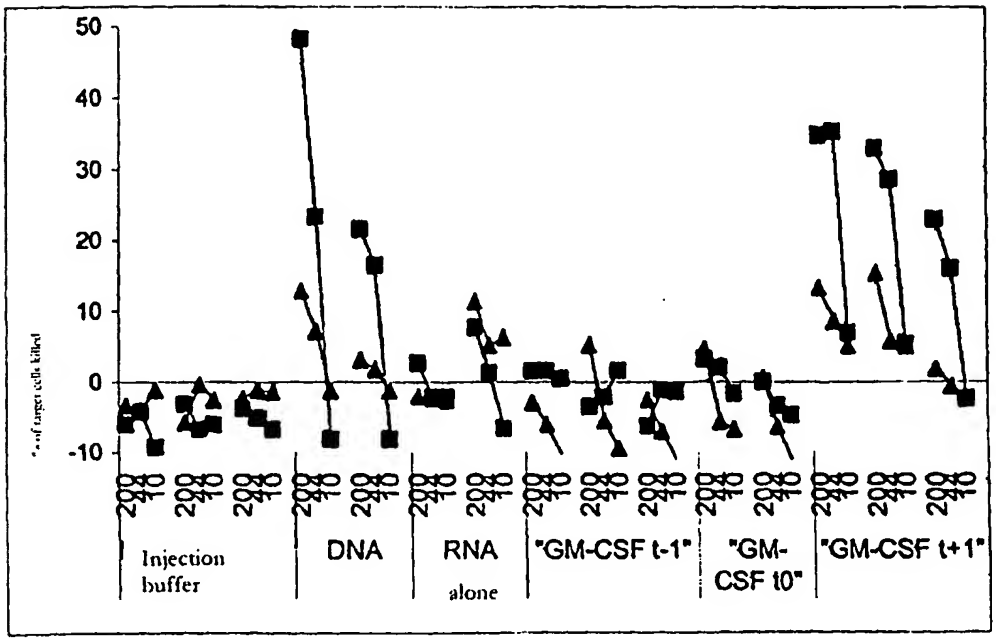

Tumour multipilicity
10
8
6
4
2
0
17
19
21
23
25
27
29
31
33
Age of the mice [weeks]
Control
Plasmid DNA
Sense RNA + protamine

Fig. 2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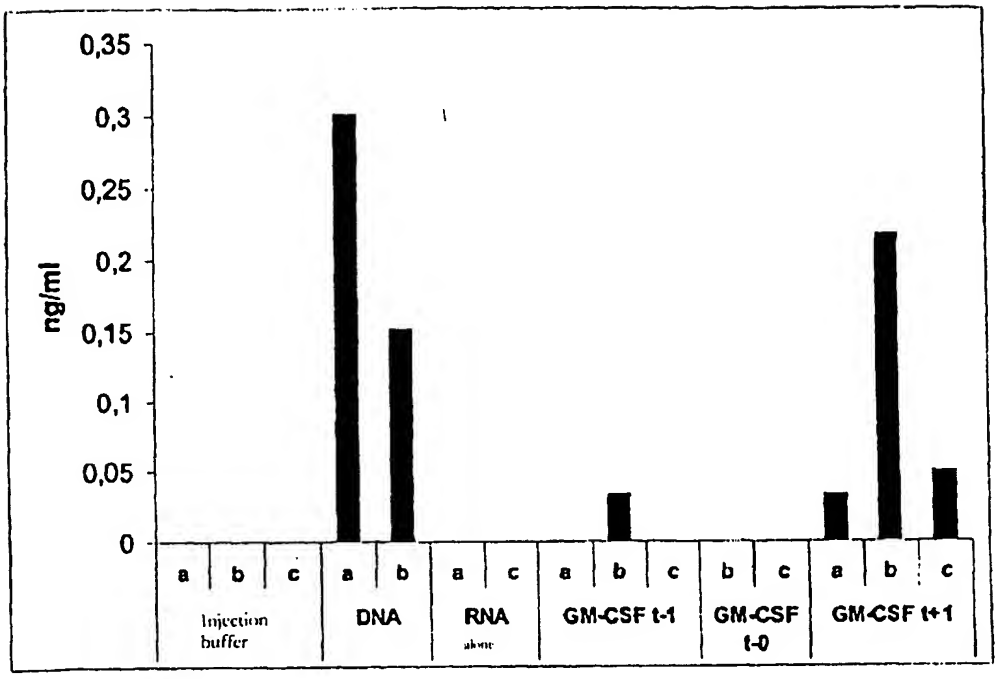
40
30
20
10
0
-10
Injection buffer
DNA
RNA alone
"GM-CSF t-1"
"GM-CSF t0"
"GM-CSF t+1"

Fig. 3

A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0,2
0,15
0,1
0,05
0
ng/ml
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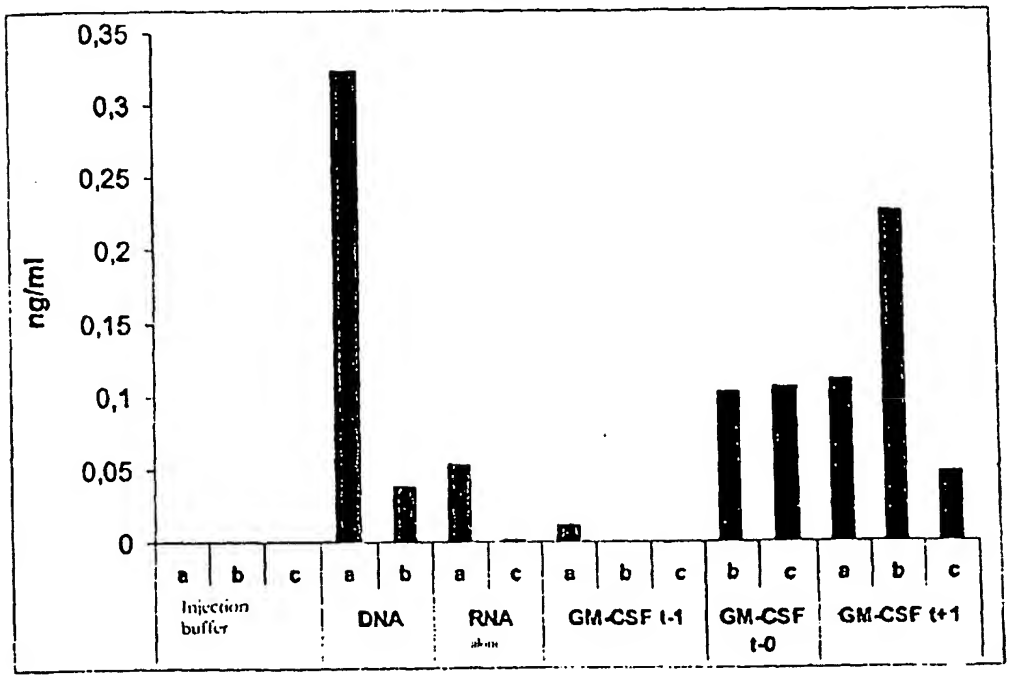
Injection buffer
DNA
RNA
GM-CSF t-1
GM-CSF t-0
GM-CSF t+1

B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ng/ml
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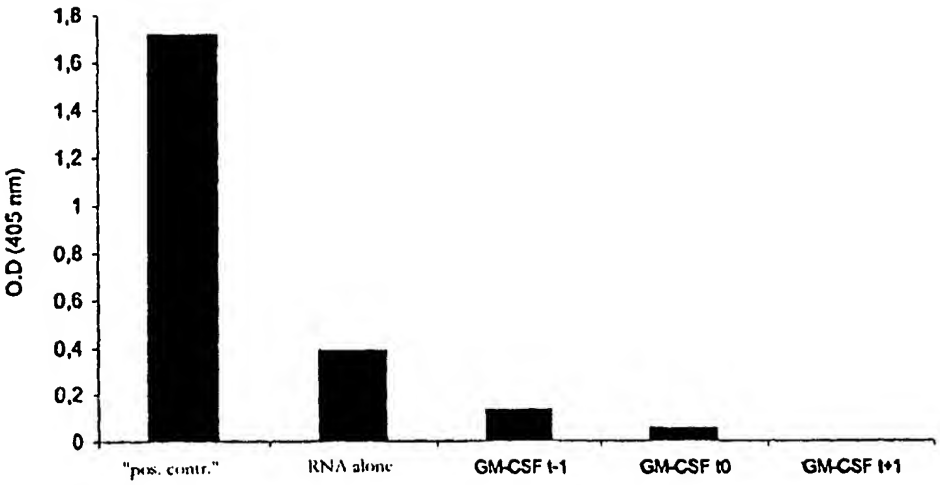
Injection buffer
DNA
RNA
GM-CSF t-1
GM-CSF t-0
GM-CSF t+1

Fig. 4

A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1,4
1,2
1
0,8
0,6
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0,2
0
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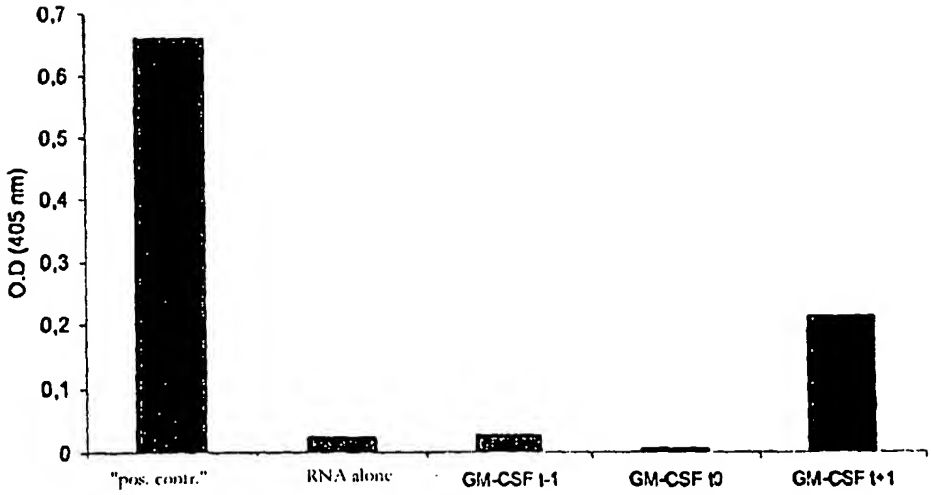
"pos. contr."
RNA alone
GM-CSF t-1
GM-CSF t0
GM-CSF t+1

B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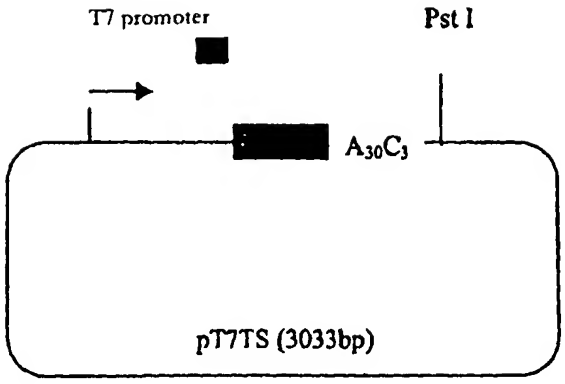
RNA alone
GM-CSF t-1
GM-CSF t0
GM-CSF t+1

Fig. 8



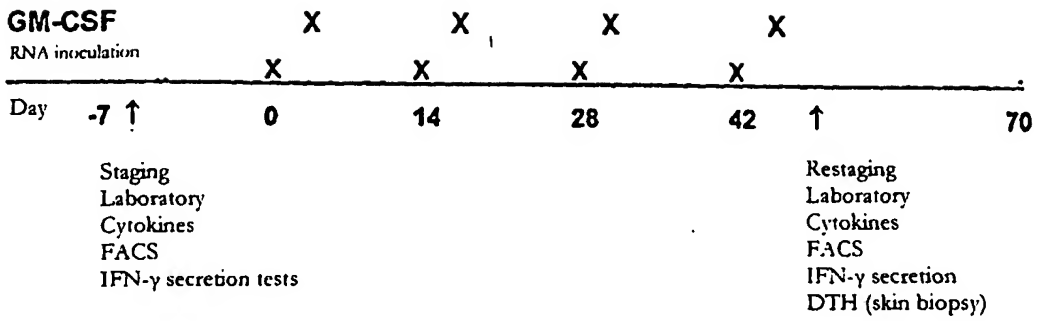
Xenopus β-globin 5' untranslated region

GCTTGTTCTTTTTGCAGAAGCTCAGAATAAACGCTCAACTTTGGC

Xenopus β-globin 3' untranslated region

GACTGACTAGGATCTGGTTACCACTAAACCAGCCTCAAGAACACCCGA
ATGGAGTCTCTAAGCTACATAATACCAACTTACACTTACAAAATGTTG
TCCCCCAAAATGTAGCCATTCGTATCTGCTCCTAATAAAAAGAAAGTT
TCTTCACATTCTA

Fig. 9


GM-CSF
RNA inoculation
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X
X
X
X
X
X
X
Day
-7
0
14
28
42
70
Staging
Laboratory
Cytokines
FACS
IFN-γ secretion tests
Restaging
Laboratory
Cytokines
FACS
IFN-γ secretion
DTH (skin biopsy)

Fig. 10

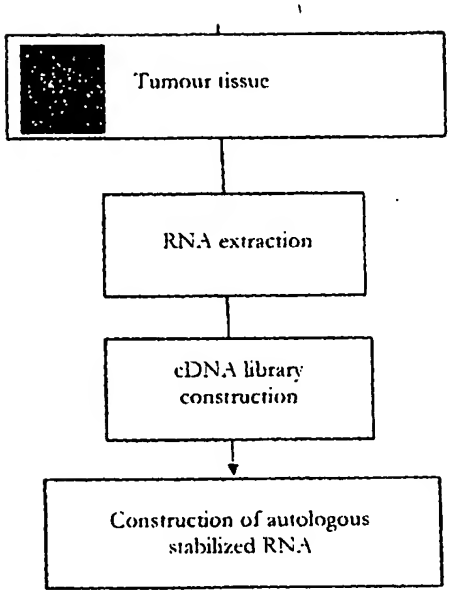

Tumour tissue
RNA extraction
cDNA library construction
Construction of autologous stabilized RNA

Fig. 11

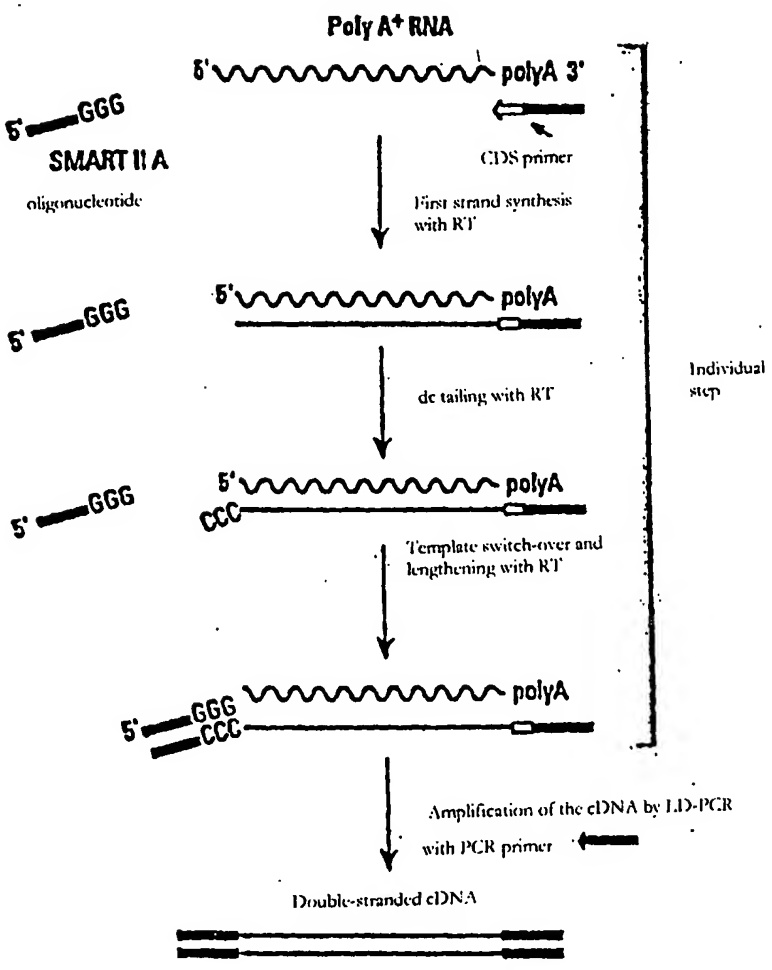
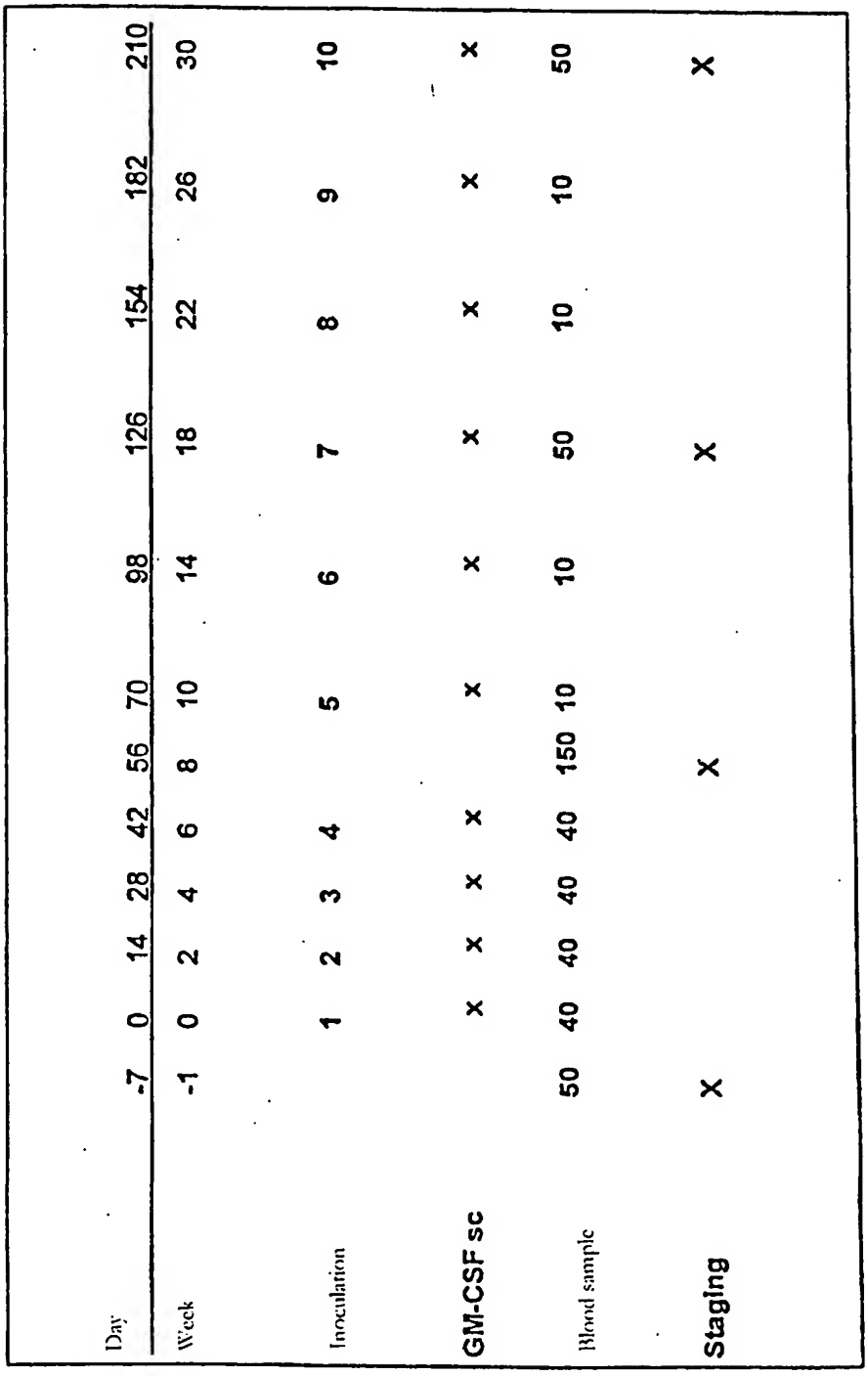

Poly A+ RNA
5'
polyA 3'
5'
GGG
SMART II A
oligonucleotide
CDS primer
First strand synthesis
with RT
5'
polyA
5'
GGG
dc tailing with RT
Individual
step
5'
polyA
5'
GGG
CCC
Template switch-over and
lengthening with RT
polyA
5'
GGG
CCC
Amplification of the cDNA by LD-PCR
with PCR primer
Double-stranded cDNA

Fig. 13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28
42
56
70
98
126
154
182
210
Week
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0
2
4
6
8
10
14
18
22
26
30
Inoculation
1
2
3
4
5
6
7
8
9
10
GM-CSF sc
x
x
x
x
x
x
x
x
x
Blood sample
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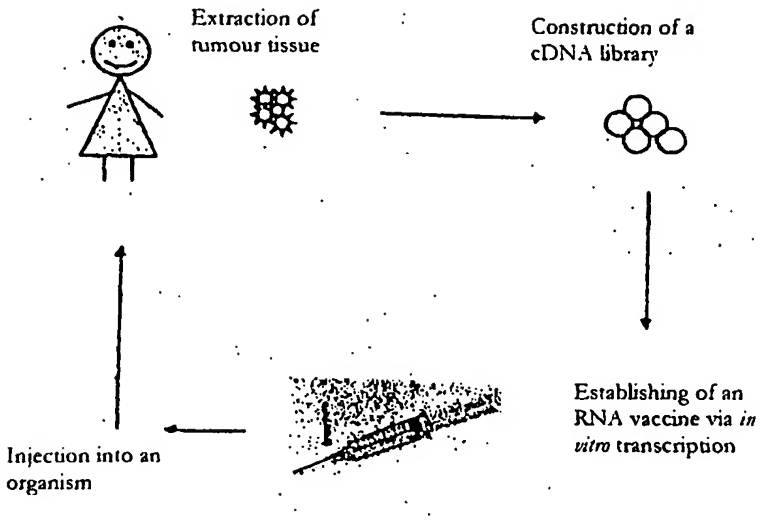
40
40
40
40
150
10
10
50
10
10
50
Staging
X
X
X

Fig. 14


Extraction of tumour tissue
Construction of a cDNA library
Establishing of an RNA vaccine via *in vitro* transcription
Injection into an organism